CHARACTERIZATION OF THE P1 PROTEIN OF THE ZUCCHINI YELLOW MOSAIC POTYVIRUS

By

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KEY TO ABBREVIATIONS

AI	amorphous inclusion
bp	base pairs
CP	capsid protein
C-terminus	carboxy-terminus
cDNA	complementary DNA
CMV	cucumber mosaic virus
CI	cylindrical inclusion
μCi	microCurie
dpm	disintegrations per minute
DIECA	diethyldithiocarbamate
ELISA	enzyme-linked immunosorbent assay
ES buffer	extraction buffer
HC/Pro	helper component/protease
kDa	kilodalton
LB	Luria broth
LDS	Laemmli dissociating solution
mw	molecular weight
N-terminus	amino-terminus
NIa	nuclear inclusion a
NIb	nuclear inclusion b
nm	nanometer
nt	nucleotide
oligo dT	oligonucleotide deoxy-thymidine
P1	P1 protein
P3	P3 protein
PCR	polymerase chain reaction
PPV	plum pox virus
PSbMV	pea seed-borne mosaic virus
PVY^N	necrotic strain of potato virus Y
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEV	tobacco etch virus
TVMV	tobacco vein mottling virus
VPg	genome-linked viral protein
WG	wheat germ
ZYFV	zucchini yellow fleck virus
ZYMV-FL/AT	aphid-transmissible isolate of zucchini yellow mosaic virus from Florida
ZYMV-CA	ZYMV isolate from California
ZYMV-MD	mild isolate of ZYMV from Florida

ZYMV-RU	ZYMV isolate from Reunion Island
ZYMV-SV	severe isolate of ZYMV from Florida

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The nucleotide sequence of the 5'-terminal P1 coding region of an aphid transmissible isolate of zucchini yellow mosaic virus (ZYMV-FL/AT) was compared to the P1 coding region of four other ZYMV isolates. Mild (ZYMV-MD) and severe (ZYMV-SV) isolates from Florida and an isolate from California (ZYMV-CA) had 95-98% homologies to ZYMV-FL/AT, whereas the P1 coding region of ZYMV from Reunion Island (ZYMV-RU) had 60% homology to ZYMV-FL/AT. The ZYMV-MD had an 18 nucleotide insert following the start codon of the P1 coding region. The P1 proteins of all ZYMV isolates shared conserved amino acids in areas of the C-terminus that have been reported to be conserved in other potyviruses.

The P1 protein from ZYMV-FL/AT was expressed in Escherichia coli and used for polyclonal antiserum production. This antiserum reacted in western blots with

extracts of pumpkin (Cucurbita pepo L.) singly infected with 22 ZYMV isolates but did not react with extracts from noninfected plants or from plants infected with three other potyviruses, a potexvirus, a cucumovirus. The P1 proteins of ZYMV isolates ranged from 33- to 35-kDa. The P1 protein of ZYMV-MD, which contained a six amino acid insert, was larger (ca. 35-kDa) than the P1 of ZYMV-FL/AT. Possible protein breakdown products (26-27-kDa) were noted for some isolates. Three isolates showed an 88-kDa product; one was tested and it reacted with antiserum to amorphous inclusion protein (AI), indicating incomplete processing between the P1 and AI cleavage site. Antiserum made to the P1 of ZYMV-RU gave similar results in western blots with respect to size heterogeneity of the P1 protein among ZYMV isolates, but it only reacted with 16 of 22 ZYMV isolates tested.

Indirect immunofluorescence tests with antisera to the P1 proteins of ZYMV-FL/AT and ZYMV-RU indicated that the P1 protein aggregates in ZYMV-infected tissues.

The P1 coding region is more variable than other regions of the genome among ZYMV isolates that have been studied. The occurrence of breakdown products, incomplete processing between P1 and AI, and different sizes of P1 proteins with certain isolates are likely due to sequence differences in the respective P1 proteins. Antisera to the P1 proteins have potential as serological probes for identifying ZYMV and distinguishing among ZYMV isolates.

CHAPTER 1
INTRODUCTION

The Potyviridae is the largest group of plant viruses, consisting of at least 180 members, most of which are aphid transmitted (Barnett, 1991). Collectively, potyviruses infect a wide range of agriculturally important crops, causing severe yield losses (Hollings and Brunt, 1981; Matthews, 1982). The potyviral genome is a message-sense, single stranded RNA molecule, consisting of about 10,000 nucleotides (nt). A characteristic feature of all potyviruses is the production of proteinaceous cylindrical inclusions (CI) in the cytoplasm of infected plants (Edwardson, 1974; Edwardson and Christie, 1991). The potyviral genome consists of a single open reading frame (ORF) which is translated into a polyprotein of about 3.5 X 10^5 kilodaltons (kDa) which is subsequently cleaved into at least eight individual proteins by viral-encoded proteases (de Mejia et al., 1985b; Carrington et al., 1989a; Dougherty and Carrington, 1988; Allison et al., 1986; Garcia et al, 1989; Verchot et al., 1991). Six of these proteins have been characterized, but little is known about two proteins referred to as P1 and P3, which are the first and third proteins encoded from the 5'-terminus of the genome. The P1

and P3 proteins flank the helper component/protease (HC/Pro), or amorphous inclusion (AI), region.

Because of the large number of potyviruses and the close relationships among many members, identification and distinction among potyviruses have received a considerable amount of attention. Properties of the capsid protein (CP) and the nucleotide (nt) sequence of the CP-encoding region have received the most attention as criteria for classification, with nt sequences of 20 distinct potyviruses and 42 strains having been determined (Ward and Shukla, 1991). This attention can be attributed to the gene encoding the CP being the first gene to be transcribed from the 3'-end of the genome by reverse transcriptase using oligo dT primers (Quemada et al., 1990; Gal-On et al., 1990), and because the CP can be extracted from virions which are easily purified from virus infected plant tissue. The CP functions to protect the viral RNA by encapsidation and it also is involved in aphid transmission (Gal-On et al., 1990; Lecoq and Purcifull, 1992). Sequence analyses have shown that a change in the amino acid triplet Asp-Ala-Gly (DAG) in the amino-terminus (N-terminus) of the CP alters aphid transmissibility (Atreya et al., 1990; 1991; Harrison and Robinson, 1988; Gal-On et al., 1990; Salomon and Raccah, 1990). The CP of two potyviruses, papaya ringspot virus type P (papaya) and soybean mosaic virus, have also been used in plant transformations to protect

against super-infection by strains of the same virus and by other potyviruses (Ling et al., 1991; Stark and Beachy, 1989).

The two nuclear inclusion proteins, NIa (49-kda) and NIb (54 to 58-kDa) aggregate in equimolar amounts in some potyviral infections, are localized in the nucleus of infected plants, and contain nuclear targeting signals (Restrepo et al., 1990). The carboxy-terminal (C-terminal) portion of the NIa is a protease responsible for processing in cis and in trans at a minimum of six sites on the polyprotein (Carrington and Dougherty, 1987a, 1987b, 1988; Carrington et al., 1988; Chang et al., 1988; Dougherty and Parks, 1989, 1991; Dougherty et al., 1988, 1989; Garcia et al., 1989; Hellmann et al., 1988; Martin et al., 1990; Riechmann et al., 1992). The NIa resembles the proteases of como-, nepo-, and picornaviruses (Domier et al., 1987; Goldbach and Wellinck, 1988), and is related to the trypsin-like family of serine proteases (Bazan and Fletterick, 1988). The N-terminal portion of the NIa is now known to be the genome-linked viral protein (VPg) attached to the 5′-terminus of the viral RNA (Siaw et al., 1985; Hari, 1981; Shahabuddin et al., 1988; Murphy et al., 1990).

The large nuclear inclusion protein, NIb, is the most highly conserved of the potyviral genes among those which have been sequenced (Ward and Shukla, 1991), and has high sequence similarity with RNA-dependent-RNA-polymerases

encoded by plus-sense RNA viruses (Domier et al., 1987; Bruenn, 1991).

The CI are produced by all members of the Potyviridae examined so far (Edwardson and Christie, 1991). They are found free in the cytoplasm (Baunoch et al., 1988), in association with the endoplasmic reticulum (Langenberg, 1986), and attached to cell membranes and plasmodesmata. Langenberg has suggested a possible involvement of CI in cell-to-cell movement of viruses because of the association of CI with virus particles and plasmodesmata (Langenberg and Purcifull, 1989; Langenberg et al., 1989). Recently, the CI have been shown to have helicase activity and to possess nucleoside triphosphate (NTP)-binding activity (Lain et al., 1988, 1989, 1990, 1991; Robaglia, et al., 1989; Riechmann et al., 1992).

The coding region between the CI and HC/Pro, designated as P3, exhibits a low sequence similarity among those potyviruses which have been sequenced. The 42-kDa P3 protein of tobacco vein mottling virus (TVMV) has been expressed in E. coli and antiserum has been prepared to it (Rodriguez-Cerezo and Shaw, 1991). In western blots, this antiserum reacted with a 42-kDa and a 37-kDa protein in infected plants indicating that there may be an alternate processing site between P3 and CI. Although it has been suggested that there is limited sequence homology between P3 and the 2A protease of picornaviruses (Domier et al., 1987;

Dougherty and Carrington, 1988), no protease activity of P3 has been demonstrated (Shukla et al., 1991).

The role of the HC/Pro in aphid transmission has been well established (Berger and Pirone, 1986; Govier and Kassanis, 1974; Pirone and Thornbury, 1983; Thornbury et al., 1985). Immunochemical studies have shown that the coding region for the amorphous inclusion (AI) is the same as the HC/Pro (Baunoch et al., 1988; de Mejia et al., 1985a), but whereas antiserum to the HC/Pro blocks aphid transmission of potato virus Y and TVMV, antiserum to the AI does not (Thornbury et al., 1985). It has been suggested that the inclusion form of this protein may be inactive in terms of aphid transmission (Dougherty and Carrington, 1988). The C-terminus of the HC/Pro functions as a protease, responsible for the cleavage between the HC/Pro and P3 (Carrington et al., 1989a, 1989b, 1990) at a conserved gly-gly (G-G) amino acid residue. Identification of two essential amino acids in the C-terminal half of the HC/Pro indicate that it is a member of the cysteine-type family of proteases (Oh and Carrington, 1989). Sequence comparisons have shown a high homology of the protease of HC/Pro with that of a dsRNA hypovirulence-associated virus of the chestnut blight fungus (Choi et al., 1991), and both proteases autocatalytically cleave between the G-G residues.

The protein encoded by the 5′-terminal region of the potyviral genome is the most variable of those which have

been sequenced (Shukla et al., 1991), and shows the greatest molecular weight (mw) variation of over 30 potyviruses which have been studied by in vitro translations (E. Hiebert, personal comm.; Hiebert and Dougherty, 1988), with a size range from 32 to 68-kDa. The C-terminus of P1 has recently been identified as a serine-type protease responsible for the autocatalytic cleavage between P1 and HC/Pro (Verchot et al., 1991). Since this cleavage occurs efficiently in the wheat germ in vitro translation system, but not in the rabbit reticulocyte lysate system, it has been suggested that an alternate cofactor may be required for the processing event. Rodriguez-Cerezo and Shaw (1991) expressed P1 in E. coli and antiserum was prepared to the expressed P1 protein. A 31-kDa protein was detected in low levels in infected tissue extracts which had been enriched for endoplasmic reticulum and mitochondria. Rodriguez-Cerezo and Shaw are the first to demonstrate the existence of both P1 and P3 in infected plants. Yeh et al. (1992), by using monoclonal antibodies (MAbs) to a 112-kDa protein product of papaya ringspot virus-type W (PRSV-W), were able to detect both 51- and 64-kDa proteins which presumably correspond to the HC/Pro and the P1 proteins, respectively.

Zucchini yellow mosaic virus (ZYMV) is one of several members of the Potyviridae which cause serious losses of cucurbitaceous crops worldwide. ZYMV was first detected in 1973 in Italy (Lisa et al., 1981), and has since been

identified in the U.S. (Adlerz et al., 1983; Purcifull et al., 1984; Provvidenti et al., 1984; Nameth et al., 1985), Israel (Antignus et al., 1989), Turkey (Davis, 1986), Japan (Suzuki et al., 1988), Australia (Greber et al., 1988), France (Lecoq et al., 1983), Lebanon (Lesemann et al., 1983), and Jordan (Al-Musa, 1989).

Both serological and biological variations have been reported for ZYMV isolates (Lisa and Lecoq, 1984; Lecoq and Purcifull, 1992; Wang et al., 1988; 1992). Serological relationships are often complex and ZYMV has been reported to cross react with watermelon mosaic virus-2 (WMV-2) in serological studies of the CP (Davis et al., 1984; Huang et al., 1986; Lisa and Lecoq, 1984; Purcifull et al., 1984; Somowiyarjo et al., 1989) and CI protein (Suzuki et al., 1988). Biological variants range from strains which induce very mild symptoms to those which induce severe and necrotic symptoms (Petersen et al., 1991; Lecoq and Purcifull, 1992). These types of variants have been detected in geographically distinct regions including France and the U.S. (Lecoq and Purcifull, 1992). Biological variants have also been observed which differ in the ability to be aphid transmitted (Lecoq et al., 1991a; Lecoq and Purcifull, 1992). Some isolates are aphid transmitted, whereas others are inefficiently transmissible or not transmitted by aphids.

Zucchini yellow mosaic virus has reportedly been responsible for severe yield losses in France (Lisa and

Lecoq, 1984), Israel (Cohen, 1986), and the U.S. (Blua and Perring, 1989). Recent studies (Lecoq et al., 1991b: Wang et al., 1991) report the use of mild isolates of ZYMV for cross protection against more severe isolates. This means of control appears promising for ZYMV. Some workers have reported that ZYMV may be transmitted at a low rate through seed (Schrijnwerkers et al., 1991; Davis and Mizuki, 1986), and many isolates are efficiently aphid transmitted.

In addition to ZYMV, several other potyviruses, such as papaya ringspot virus type-W (PRSV-W), watermelon mosaic virus-2 (WMV-2), zucchini yellow fleck virus (ZYFV), bean yellow mosaic virus, Bryonia mottle virus (Lovisolo, 1980), and Telfairia mosaic virus (Shoyinka et al., 1987) have been reported to infect cucurbits in the field. As a result, the possibility for genetic recombination among these cucurbit potyviruses exists (King, 1987; Morozov et al., 1989). Consequently, the ability to accurately diagnose and differentiate isolates is important in breeding programs designed to control ZYMV through the development and use of resistant cucurbit cultivars. Differentiation of ZYMV isolates is also important in cross-protection studies.

Diagnosis and distinction of ZYMV isolates have been based in part on differential host reactions. Immunochemical assays involving both polyclonal (PAb) and monoclonal (MAb) antisera to the CP are useful for diagnosis

of ZYMV, but are not completely definitive for distinction between isolates.

Studies of both structural and nonstructural proteins of potyviruses have been based on their ease of isolation, either because they are readily purified, they aggregate in infected tissue, or, as with the CP, they can be easily cloned by oligo dT priming. Neither P1 nor P3 have been shown to accumulate or aggregate in infected plants and thus they have not been well characterized. As an early part of this study, several MAbs to the CP of ZYMV were evaluated for their ability to distinguish and diagnose ZYMV isolates, as described in Appendix 1. The primary focus of this study, however, was to further characterize the P1 and P3 of ZYMV. To achieve this goal, the objectives of this study included (1) characterization of the P1 and P3 proteins and coding regions of an aphid-transmissible isolate of ZYMV from Florida (ZYMV-FL/AT); (2) comparison of the P1 and P3 proteins and their coding regions from ZYMV-FL/AT to those of other ZYMV isolates and other cucurbit potyviruses; (3) evaluation of antisera to these proteins as serological probes for studying the variability of ZYMV isolates; and (4) evaluation of the antigenic relationships of these proteins among ZYMV isolates and other potyviruses infecting the Cucurbitaceae. The high sequence variability of P1 and P3 make both of them interesting protein coding regions for study, and knowledge gained in this research should lead to

a better understanding of the roles of P1 and P3 in the viral infection process.

This study reports nucleotide sequence variability among the P1 coding region of five ZYMV isolates. Antisera to the P1 proteins of two ZYMV isolates detected size differences in the P1 proteins, incompletely processed P1 products, possible breakdown products, and antigenic differences among several ZYMV isolates. Nucleotide sequence information only is presented for the P3 coding region of ZYMV-FL/AT due to the inability to express this protein in E. coli as a result of its apparent toxicity.

CHAPTER 2
CLONING AND SEQUENCING OF A FLORIDA ISOLATE OF ZUCCHINI YELLOW MOSAIC VIRUS

Introduction

It has been hypothesized that potyvirus strains "show an overall high sequence identity irrespective of the gene product being considered, while distinct viruses have a significantly lower degree of identity between gene products" (Shukla et al., 1991, p.181). The nucleotide (nt) sequence identities for the CP gene of distinct potyviruses range approximately from 30 to 60%, while the nucleic acid sequence identities determined for strains of a potyvirus are greater than 95% (Ward and Shukla, 1991). These authors point out that for the four potyviruses that have been sequenced, the P1 coding region of the potyviral genome has a lower homology than other coding regions, and that this appears to be the most variable region of the genome. They also suggest that this variability may indicate a possible role for P1 in specific virus-host interactions.

The major goal in this study was to characterize and compare the P1 and P3 proteins of several ZYMV isolates. A means of achieving this goal was to produce P1 and P3 of ZYMV-FL/AT in a high level expression system, and produce

specific antisera to each. The first step in this process was to clone the ZYMV genome and determine the nt and deduced amino acid sequence of the region encoding P1 through P3.

Materials and Methods

Virus Isolates

An isolate of ZYMV from Florida (Purcifull et al., 1984), which has been maintained in a greenhouse by aphid transmission for the past three years, FC-1119AT, was used as the type isolate throughout this study, and it will be referred to as ZYMV-FL/AT. The sequence of P1 through P3 of ZYMV-FL/AT was compared to a ZYMV isolate from California (ZYMV-CA) which was sequenced by R.Balint (personal comm.), one from Reunion Island (ZYMV-RU, Baker et al., 1991b), a severe, necrotic isolate from Florida (FC-2088) designated as ZYMV-FL/SV, and a mild isolate from Florida (FC-1994) designated as ZYMV/MD.

Virus Purification and RNA Extraction

The protocol used for virus purification is similar to that described by Lecoq and Pitrat (1985). ZYMV-infected Cucurbita pepo L. 'Small Sugar' tissue was harvested 14 days post inoculation and homogenized for 10 sec with 3.75 volumes of 0.3 M K_2HPO_4, pH 8.5, with freshly added 0.2% sodium diethyldithiocarbamate (DIECA) and 0.2% 2-mercaptoethanol. The resulting slurry was then homogenized with 2/3 volume of trichlorofluorethane (Freon) for one min.

After centrifugation at 5,000 *g* for 10 min, the aqueous phase was made 1% with Triton X-100 and was stirred for 20 min at 4 C. Partially purified virus was collected by ultracentri-fugation at 37,000 rpm in a Beckman Ti70 rotor for 1.5 hr at 4 C and was resuspended in 0.02 M HEPES, pH 8.2 with a tissue homogenizer. After stirring for one hr at 4 C, the suspension was partially clarified by centrifugation at 2,500 *g* for 2 min. The supernatant was layered onto a Cs_2SO_4 gradient (10 g/27 ml 0.01 M HEPES, pH 8.2) and centrifuged for 16 hr at 32,000 *g* in a Beckman SW41 rotor at 4 C. The opalescent virus zone 24-27 mm from the bottom of the tube was collected, diluted with one volume of 0.02 M HEPES, pH 8.2, and was centrifuged at 10,000 *g* for 10 min. The supernate was made 8% with polyethylene glycol (PEG) 8000 and was stirred for 20 min at 4 C. A pellet containing virus was collected by centrifugation at 10,000 *g* for 10 min, and resuspended in 0.02 M HEPES, pH 8.2. Concentrations of the virus preparations were estimated by spectrophotometry using an approximate extinction coefficient of A_{260}=2.5 (1 mg/ml, 1 cm light path). Preparations were divided into aliquots and stored at -80 C.

A virus preparation containing approximately 3 mg/ml was added to an equal volume (1 ml) of RNA dissociating solution [200 mM Tris-HCl, 2 mM EDTA, 2% sodium dodecyl sulfate (SDS), pH 9.0], and six μl of protease K. After a 10 min incubation at room temperature (RT), the preparation

was layered onto a linear-log sucrose density gradient (Brakke and Van Pelt, 1970) and was subjected to ultracentrifugation at 39,000 rpm for 5 hr at 15 C with a Beckman SW41 rotor. Gradient zones containing RNA were collected using an ISCO UV analyzer. The RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2-5 volumes of 100% ethanol (Sambrook et al., 1989). Pellets were resuspended in 50 μl diethyl- pyrocarbonate (DEPC) treated water and stored at -80 C.

Synthesis of cDNA

The initial cDNA library of the ZYMV RNA was made using λgt11 (Lambda Librarian, Stratagene, LaJolla, CA), which is based on a modification of a procedure described by Gubler and Hoffman (1983). Freshly prepared viral RNA was used for cloning, with 8.3 μg RNA as the template in the first strand synthesis reaction. Both random and oligo dT primers were used in two separate first strand synthesis reactions which were labeled by the addition of [^{32}P]dCTP. Incorporation of label was measured in fractions eluted from P-60 columns (100-200 mesh Bio-Gel P-60; Bio-Rad, Melville, NY). Those fractions with counts between 2,000 and 30,000 disintegrations per minute (dpm) were combined, and nucleic acid was precipitated with ethanol. Second strand synthesis and the ligation of EcoRI/NotI linkers were performed according to the manufacturer's instructions.

Size analysis of cDNA was performed on a 0.9% agarose gel. The gel was exposed to X-ray film and compared to a 1-kb ladder molecular weight standard (BRL, Gaithersburg, MD). A zone corresponding to 800-7,000 base pairs (bp) was cut from the gel and electroeluted with a Bio-Rad (Melville, NY) electroeluter according to manufacturer's instructions. The volume of the eluted cDNA was reduced with water-saturated *n*-butanol, followed by an ethanol precipitation. The cDNA preparations with linkers from both random and oligo dT priming were combined and they were ligated to EcoR1-digested, calf intestinal alkaline phosphatase (CIAP)-treated λgt11 DNA (Protoclone λgt11 System, Promega, Madison, WI) for three hr at 22 C (Huynh et al., 1984.). Molar ratios of vector to insert ranged from 1:1 to 1:2 to 1:3. The ligated cDNA was packaged and titered according to manufacturer's instructions using the Packagene Lambda DNA Packaging System (Promega, Madison, WI). Packaged phage was titered on Escherichia coli strain Y1090 grown from a single colony to an $O.D._{600}$ of 0.6 to 0.8 in Luria broth (LB) (Sambrook et al., 1989), supplemented with 10 mM $MgSO_4$ and 0.2% maltose.

A library made specifically to the 5'-terminus of the ZYMV genome was constructed using the Lambda ZAP II/EcoRI Cloning Kit (Stratagene, La Jolla, CA). A primer, with the sequence of 5'-CGGTGTGTGCGCTAC-3', which corresponded to an area encoding the cylindrical inclusion (CI) protein was

used in this cloning experiment and was synthesized at the University of Florida DNA Synthesis Core. The host strain used for this vector system was E. coli XL1-Blue.

Nucleic Acid Hybridization of ZYMV Clones

Nucleic acid probes prepared from viral RNA were labeled with [^{32}P]dCTP using either a Nick Translation Kit (Promega, Madison, WI) or a Random Primed DNA Labeling Kit (Boehringer Mannheim (Indianapolis, IN) according to manufacturer's instructions. Nylon Hybond membranes, 0.45 μ pore size (Amersham, Inc., Arlington Heights, IL) cut to fit media plates containing plaques, were laid onto the agar surface for approximately 15 min. Membranes were then placed onto filter paper pads soaked in three solutions sequentially for 15 min each: (1) 0.5 M NaOH, 1.5 M NaCl, (2) 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, and (3) 2X SSC (Sambrook et al., 1989). They were placed in a UV Crosslinker for 2 min, after which they were prehybridized in 10 ml of 1% SDS, 1 M NaCl, 10% PEG 8000, with 5 mg/ml denatured salmon sperm. Membranes were placed in a heat seal bag and incubated at 55 C for 15 min with agitation, after which 100-200 μCi/ml denatured probe was added. This was followed by an overnight incubation at 55 C. Membranes were washed twice in 100 ml of 2X SSC for 5 min each at room temperature (RT), followed by two rinses in 200 ml of 2X SSC containing 1% SDS at 55 C for 30 min each. A final rinse was in 100 ml of 0.1X SSC at RT for 30 min. Washed

membranes were exposed to X-ray film with an intensifying screen.

Immunoscreening of ZYMV Clones

Immunoscreening for clones expressing specific regions of the ZYMV genome was conducted essentially according to manufacturer's instructions as described in the picoBlue Immunoscreening Kit (Stratagene) and by Short et al., (1988). Recombinant bacteriophage library lysate was added to 200 μl of the appropriate strain of E. coli cells, and allowed to absorb at 37 C for 15 min. This was added to 3 ml of LB soft agar containing 10 mM $MgSO_4$, which was poured onto LB plates and incubated at 42 C for 3-4 hr until plaques appeared. A nitrocellulose membrane (NCM) which had previously been saturated with 10 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) was laid onto each plate. The IPTG is a gratuitous inducer used to induce the expression of the ß-galactosidase fusion protein. Plates were then moved to 37 C and allowed to incubate an additional 4-6 hr. Membranes were removed, rinsed three times for 5 min in a solution containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 (TBST), and then incubated in TBST containing 5% Carnation dry milk powder for 15 min to block protein binding sites. E. coli lysate was prepared as described in Huynh et al., (1984) and added at 1 mg/ml to the blocking solution. Antisera to the CP and CI of ZYMV, to the small nuclear inclusion protein (NIa) of tobacco etch virus (TEV),

the HC/Pro of TVMV, and to the AI of papaya ringspot virus type-W (PRSV-W) were used for primary antibody screening. The virus antisera used in screening were known to cross-react with corresponding proteins of ZYMV and other potyviruses. Primary antibody was diluted in the blocking solution at 1/500 to 1/1000 and incubated overnight at 4 C with shaking. Each step was followed by three 5 min washes with TBST. The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO), was added at a dilution of 1/2000 in blocking solution and was incubated with shaking at RT for 1-2 hr. After rinsing in TBST, a final rinse was made in development buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.6.). The color substrates (Gibco BRL, Gaithersburg, MD), nitroblue tetrazolium (NBT) (22 μl of a 75 mg/ml solution) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (20 μl of a 50 mg/ml solution) were added to 20 ml development buffer with 50 μl of 2M $MgCl_2$. Color development was allowed to proceed for approximately 30 min before reactions were stopped by rinsing with deionized water. Positive clones were isolated with a sterile pipette and placed in a buffer containing 100 mM NaCl, 50 mM Tris, pH 7.5, 10 mM $MgSO_4$ (SM buffer) with 5% chloroform and stored at 4 C. Clones were purified by 2 to 3 rounds of plating and screening.

Analysis of ZYMV Clones

Plaque purified clones in λgt11 which were positive in immunoscreening were raised in a 5 ml culture in the lysogenic host E. coli Y1089 and were prepared by a mini-prep procedure. Preparations were digested with EcoRI, run on an agarose gel, transferred to a nylon membrane using an agarose gel transfer unit (Millipore MilliBlot-V System, Bedford, MA), and exposed to a [^{32}P]dCTP labeled ZYMV probe.

Clones in both λgt11 and λZAPII were analyzed by polymerase chain reaction (PCR) using λgt11 forward and reverse primers. Plaques were placed into 20 μl of SM buffer and frozen at -20 C. Ten μl were used as the template in a BIOS thermocycler with three cycles of 94 C for 3 min, 45 C for 1 min, and 72 C for 3 min. This was followed by 35 cycles at 93 C for 1 min, 45 C for 1 min, and 72 C for 3 min. Plasmid clones were analyzed with PCR by placing individual colonies in 20 μl of 20 mM Tris-HCl, pH 8, containing 1% Triton X-100, heating to 95 C for 10 min, and centrifuging at 10,000 *g* for 2 min. Ten μl of this template was used in PCR with the appropriate primers.

Subcloning of Recombinant Bacteriophage

Clones identified in λgt11 were digested as described, and extracted from an agarose gel using a Prep-A-Gene DNA Purification Kit (Bio-Rad). Fragments were then subcloned by ligating into EcoR1 digested pGEMEX-1 (Promega) which had been treated with CIAP (Sambrook et al., 1989). Plasmid

clones (pBluescript SK-) were isolated from λZAP II with the use of the helper phage R408 according to manufacturer's instructions (Stratagene). Recombinant plasmid cultures were prepared for further analysis by the alkaline lysis miniprep procedure (Sambrook et al., 1989).

Two additional P1-encoding regions, one from ZYMV-SV and one from ZYMV-MD, were cloned into the pETh vector (McCarty et al., 1991) after increase of cDNA by PCR using primers specific for P1, following procedures similar to those described by Robertson et al. (1991).

DNA Sequencing of ZYMV Clones

The nucleotide sequences of plasmid preparations and PCR products were determined using the standard Sanger dideoxy chain termination method (1977) employed in both US Biochemical Corp., Cleveland, OH, and Pharmacia LKB, Piscataway, NJ, sequencing kits. Both 6% and 4% acrylamide gels with 7 M urea were used. Sequence analysis and comparisons were made using the University of Wisconsin Genetics Computer Group Sequence Software (GCG) available at the University of Florida ICBR Biological Computing Facility.

Results

Clones Representing the ZYMV Genome

The initial cloning experiment using λgt11 yielded an estimated 73 x 10^6 clear (positive) plaques, and the yield

from λZAPII was estimated to be 14 x 10^6 clear plaques. After serological screening with antisera to the CP, NIa, CI, AI, and HC/Pro, several clones were selected which were specific to each of these protein-encoding regions on the ZYMV genome. In the λgt11 cloning experiment, ten clones were identified by the reactivity of their expressed products with CP antiserum. Nine of these clones were specific for the CP and an additional, large clone of 4.5-kilobase pairs (kbp), was reactive with both CP and NIa antisera (Table 2-1). Two clones were identified which represented the CI, and two which represented the AI coding region. Preliminary sequencing indicated that the AI-positive clones were not large enough to include the P1-encoding region. Subsequent cloning experiments using λZAPII yielded six additional CI-positive clones, seven additional AI-positive clones, and three clones that were both CI- and AI-positive (Table 2-2). Three of the CI clones were large enough to code for both the CI and AI regions, and their products reacted with antisera to both CI and AI proteins. An AI-positive clone of 2.9-kbp was selected (AI6) which included all of P1, AI, and a portion (ca. 600-bp) of P3. Clone AI6 also represented 55-bp of the leader sequence. None of the 30 clones was identified that represented the entire leader sequence, which, based on the ZYMV-CA sequence, is approximately 141-bp.

Table 2-1. ZYMV-FL/AT cDNA clones identified by immunoscreening in λgt11.

Clone designation	Approximate size (kbp)	Serological reactivity[a]
CP1	0.85	CP
CP2	1.40	CP
CP3	0.60	CP
CP4	0.40	CP
CP5	1.75	CP
CP6	0.75	CP
CP7	4.50	CP,NIa
CP8	1.80	CP
CP9	3.30	CP
CP10	1.30	CP
CI1	2.30	CI
CI2	2.20	CI
AI1	1.60	AI
AI2	0.90	AI

[a] coding regions listed refer to clones, the products of which reacted with antisera to the capsid protein (CP), small nuclear inclusion protein (NIa), cylindrical inclusion protein (CI), or amorphous inclusion protein (AI) in immunoscreening assays.

Table 2-2. ZYMV-FL/AT cDNA clones identified by immunoscreening in λZAPII.

Clone designation	Approximate size (kbp)	Serological[a] reactivity
CI3	1.80	CI
CI4	3.50	CI,AI
CI5	3.00	CI,AI
CI6	2.90	CI,AI
CI7	2.80	CI
CI8	2.00	CI
CI9	1.60	CI
CI10	1.80	CI
CI11	2.50	CI
AI3	1.50	AI
AI4	1.60	AI
AI5	1.90	AI
AI6	2.90	AI
AI7	1.50	AI
AI8	3.00	AI
AI9	3.00	AI

[a] coding regions listed refer to clones, the products of which reacted with antisera to the cylindrical inclusion protein (CI), or amorphous inclusion protein (AI) in immunoscreening assays.

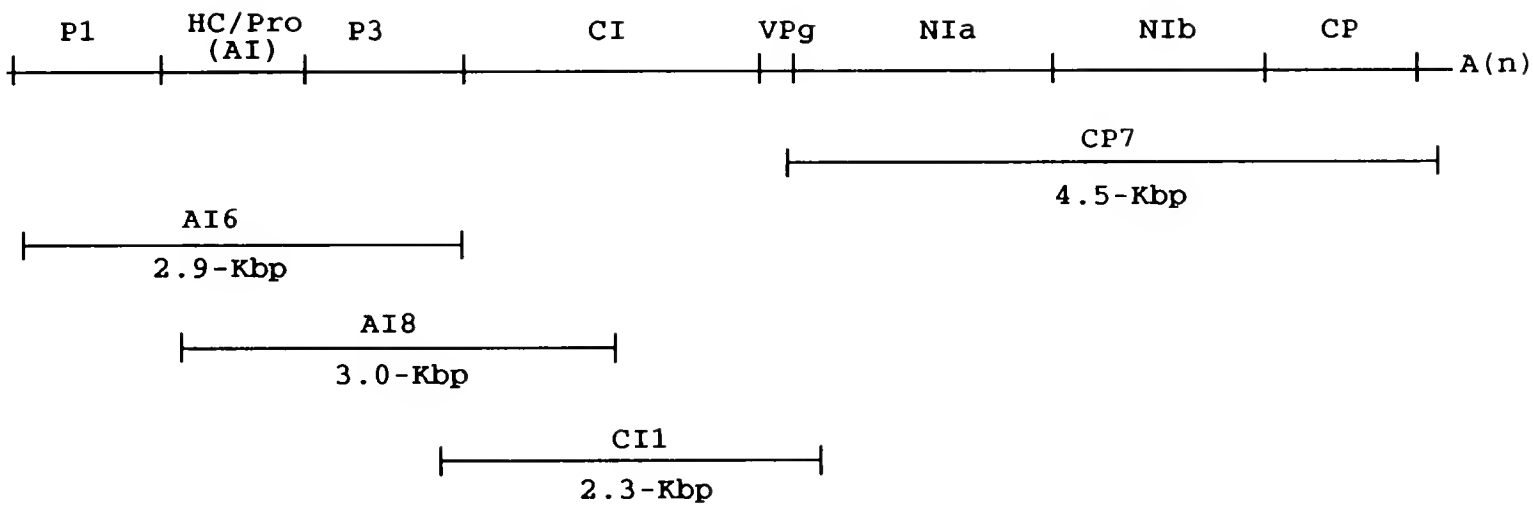

Fig. 2-1. Map of selected cDNA clones from λgt11 and λZAPII which represent the genome of ZYMV-FL/AT.

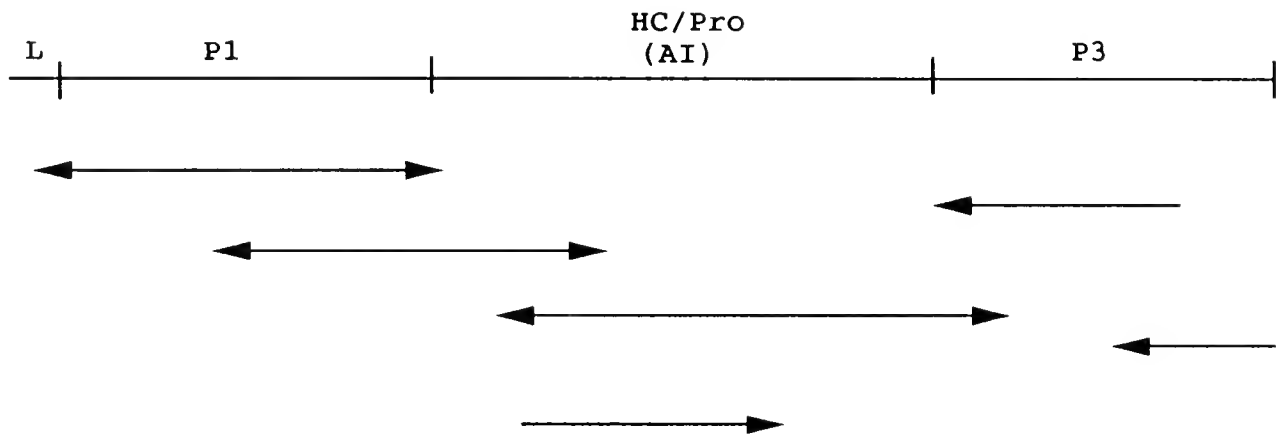

Fig. 2-2. Sequencing strategy used for the cDNA clones representing P1-, HC/Pro(AI)-, and P3-encoding regions. Arrows indicate the direction of sequencing and distance read from the beginning of the clone or from the location of a primer. L=leader sequence.

Sequences Representing the 5'-terminus of ZYMV

Four clones were selected which together represent the entire ZYMV-FL/AT genome; AI6 (2.9-kbp), AI8 (3.0-kbp), CI1 (2.3-kbp), and CP7 (4.5-kbp) (Fig. 2-1). Several additional clones were used to sequence the entire region from P1 through the P3-encoding region (Fig. 2-2).

The sequence of ZYMV-FL/AT, from the 55-bp of the leader through P3, with the corresponding amino acid sequence, is presented in Fig. 2-3. Based on consensus sequences (Oh and Carrington, 1989; Mavankal & Rhoads, 1991; Thornbury et al., 1990; Verchot et al., 1991), the P1-encoding region of ZYMV-FL/AT is 912-bp, the AI is 1,386-bp, and P3 is 1,191-bp in length. The cleavage site between P1 and AI is at amino acid position 304-305 (tyr/ser), whereas the site between AI and P3 is at amino acid position 766-767 (gly/gly), and between P3 and CI is at position 1,164-1,165 (glu/gly).

In the P1-encoding region, the consensus sequence for the predicted protease at the C-terminus is Gly-Xaa-Ser-Gly---Phe-Ile-Val-Arg-Gly (Verchot et al., 1991), Xaa being any amino acid, whereas that for ZYMV-FL/AT is Gly-Cys-Ser-Gly---Leu-Val-Ile-Arg-Gly. In addition, Ser and His were found at positions 264 and 223, respectively. These amino acids are strictly conserved among potyviruses. Both the Ser and His have been shown by point mutations in tobacco etch virus

Fig. 2-3. Nucleotide and deduced amino acid sequence of ZYMV-FL/AT. Amino acids are represented by a single letter code. Underlined residues represent sequences similar to conserved potyviral sequences. Asterisks indicate amino acids strictly conserved among potyviruses that have been sequenced. Slash(/) indicates cleavage sites between proteins.

```
1                                                                   60
AACTCTTACAGTATTTAGAAATTCTCCAATCACTTCGTTTACTTCAGACATAACAATGGC
                                                                    *
                                                                M   A
61                                                                 120
CTCTATCATGATTGGTTCAATCTCCGTACCCATTGCAAAGACTAAGCAGTGTGCAAACAC
 S  I  M  I  G  S  I  S  V  P  I  A  K  T  K  Q  C  A  N  T
121                                                                180
TCAAGTAAGTAATCGGGTTAATATAGTGGCACCTGGCCACATGGCAACATGCCCATTGCC
 Q  V  S  N  R  V  N  I  V  A  P  G  H  M  A  T  C  P  L  P
181                                                                240
ACTGAAAACGCACATGTATTACAGGCATGAGTCCAAGAAGTTGATGCAATCAAACAAAAG
 L  K  T  H  M  Y  Y  R  H  E  S  K  K  L  M  Q  S  N  K  S
241                                                                300
CATTGACATTCTGAACAATTTCTTCAGCACTGACGAGATGAAGTTTAGGCTCACTCGAAA
 I  D  I  L  N  N  F  F  S  T  D  E  M  K  F  R  L  T  R  N
301                                                                360
CGAGATGAGCAAGGTGAAAAAGGGTCCGAGTGGGAGGATAGTCCTCCGCAAGCCGAGTAA
 E  M  S  K  V  K  K  G  P  S  G  R  I  V  L  R  K  P  S  K
361                                                                420
GCAGCGGGTTTTCGCTCGTATTGAGCAGGATGAGGCAGCACGCAAGAAAGAGACTGTTTT
 Q  R  V  F  A  R  I  E  Q  D  E  A  A  R  K  K  E  T  V  F
421                                                                480
CCTCGAAGGAAATTATGACGATTCTATCACAAATCTAGCACGTGTTCTTCCACCTGAAGT
 L  E  G  N  Y  D  D  S  I  T  N  L  A  R  V  L  P  P  E  V
```

Fig. 2-3--continued.

```
481                                                                 540
GACTCACAACGTTGATGTGAGCTTGACGTCATCGTTTTACAAGCGCACATACAAGAAGGA
  T  H  N  V  D  V  S  L  T  S  S  F  Y  K  R  T  Y  K  K  E
541                                                                 600
AAGGAAGAAAGTGGCGCAAAAGCAAATTGTGCAAGCACCACTCAATAGCTTGTGCACACG
 R  K  K  V  A  Q  K  Q  I  V  Q  A  P  L  N  S  L  C  T  R
601                                                                 660
TGTTCTTAAAATTGCACGCAATAAAAATATCCCTGTTGAGATGATTGGCAACAAGAAGGC
  V  L  K  I  A  R  N  K  N  I  P  V  E  M  I  G  N  K  K  A
661                                                                 720
GAGACATACACTCACCTTCAAGAGGTTTAGGGGATATTTTGTTGGAAAGGTGTCAGTTGC
  R  H  T  L  T  F  K  R  F  R  G  Y  F  V  G  K  V  S  V  A
 721                                                                 780
GCATGAAGAAGGACGAATGCGGCGCACTGAGATGTCGTATGAGCAGTTTAAATGGATTCT
 *
 H  E  E  G  R  M  R  R  T  E  M  S  Y  E  Q  F  K  W  I  L
781                                                                 840
AAAAGCCATTTGTCAGGTCACCCATACAGAGCGAATTCGTGAGGAAGATATTAAACCAGG
  K  A  I  C  Q  V  T  H  T  E  R  I  R  E  E  D  I  K  P  G
841                                                                 900
TTGTAGTGGGTGGGTGTTGGGCACTAATCATACATTGACTAAAAGATATTCAAGATTGCC
    *
 C  S  G  W  V  L  G  T  N  H  T  L  T  K  R  Y  S  R  L  P
```

Fig. 2-3--continued.

901 960

ACATTTGGTGATTCGAGGTAGAGACGACGATGGGATTGTGAACGCGCTGGAACAGGTGTT

H L V I R G R D D D G I V N A L E Q V L

961 1020

ATTTTATAGCGAAGTTGACCACTATTCGTCGCAACCGGAAGTTCAGTTCTTCCAAGGATG

F Y/ S E V D H Y S S Q P E V Q F F Q G W

1021 1080

GCGACGAATGTTTGACAAGTTTAGGCCCAGCCCAGATCATGTGTGCAAAGTTGACCACAA

R R M F D K F R P S P D H V C K V D H N

1081 1140

CAACGAGGAATGTGGTGAGTTAGCAGCAATCTTTTGTCAGGCTTTATTCCCAGTAGTGAA

*

N E E C G E L A A I F C Q A L F P V V K

1141 1200

ACTATCGTGCCAAACATGCAGAGAAAGCTTAGTAGAAGTTAGCTTTCGAGGAATTAAAGA

L S C Q T C R E S L V E V S F R G I K D

1201 1260

TTCTTTGAACGCAAACTTTATTGTCCACAAGGATGAATGGGGTAGTTTCAAGGAAGGCTA

S L N A N F I V H K D E W G S F K E G Y

1261 1320

TCAATACGATAATATTTTCAAATTAATCAAAGTGGCAACACAGGCAACTCAGAATCTCAA

Q Y D N I F K L I K V A T Q A T Q N L K

1321 1380

GCTCTCATCTGAAGTTATGAAATTAGTTCAGAACCACACAAGCACTCACATGAAGCAAAT

L S S E V M K L V Q N H T S T H M K Q I

Fig. 2-3--continued.

1381 1440

ACAAGACATCAACAAGGCGCTCATGAAAGGTTCATTGGTTACGCAAGACGAATTGGACTT

Q D I N K A L M K G S L V T Q D E L D L

1441 1500

AGCTTTGAAACAGCTTCTTGAAATGACTCAGTGGTTTAAGAACCACATGCACCTGACTGG

A L K Q L L E M T Q W F K N H M H L T G

1501 1560

TGAGGAGGCATTGAAGATGTTCAGAAATAAGCGTTCTAGCAAGGCCATGATAAATCCTAG

E E A L K M F R N K R S S K A M I N P S

1561 1620

CCTTCTATGTGACAACCAATTGGACAAAAATGGAAATTTTGTTTGGGGAGAAAGAGGATA

L L C D N Q L D K N G N F V W G E R G Y

1621 1680

CCATTCCAAGCGATTATTCAAGAACTTCTTCGAAGAAGTAATACCAAGCGAAGGATATAC

H S K R L F K N F F E E V I P S E G Y T

1681 1740

GAAGTACGTAGTGCGAAACTTTCCAAATGGTACTCGTAAGTTGGCCATAGGCTCGTTGAT

K Y V V R N F P N G T R K L A I G S L I

1741 1800

TGTACCACTCAATTTGGATAGGGCACGCACTGCACTACTTGGAGAGAGTATTGAGAAGAA

V P L N L D R A R T A L L G E S I E K K

1801 1860

GCCACTCACATCAGCGTGTGTCTCCCAACAGAATGGAAATTATATACACTCATGCTGCTG

P L T S A C V S Q Q N G N Y I H S C C C

Fig. 2-3--continued.

```
1861                                                          1920
TGTAACGATGGATGATGGAACCCCGATGTACTCCGAGCTTAAGAGCCCGACGAAGAGGCA
  V  T  M  D  D  G  T  P  M  Y  S  E  L  K  S  P  T  K  R  H
1921                                                          1980
TCTAGTTATAGGAGCTTCTGGTGATCCAAAGTACATTGATCTGCCAGCATCTGAGGCAGA
  L  V  I  G  A  S  G  D  P  K  Y  I  D  L  P  A  S  E  A  E
1981                                                          2040
ACGCATGTATATAGCAAAGGAAGGTTATTGCTATCTCAATATTTTCCTCGCAATGCTTGT
                              *
 R  M  Y  I  A  K  E  G  Y  C  Y  L  N  I  F  L  A  M  L  V
2041                                                          2100
AAATGTTAATGAGAACGAAGCAAAGGATTTCACCAAAATGATTCGTGATGTTTTGATCCC
  N  V  N  E  N  E  A  K  D  F  T  K  M  I  R  D  V  L  I  P
2101                                                          2160
CATGCTTGGGCAGTGGCCTTCATTGATGGATGTTGCAACTGCAGCATATATTCTAGGTGT
  M  L  G  Q  W  P  S  L  M  D  V  A  T  A  A  Y  I  L  G  V
2161                                                          2220
ATTCCATCCTGAAACGCGATGCGCTGAATTACCCAGGATCCTTGTTGACCACGCTACACA
  F  H  P  E  T  R  C  A  E  L  P  R  I  L  V  D  H  A  T  Q
2221                                                          2280
AACCATGCATGTCATTGATTCTTATGGATCACTAACTGTTGGGTATCACGTGCTCAAGGC
      *
 T  M  H  V  I  D  S  Y  G  S  L  T  V  G  Y  H  V  L  K  A
```

Fig. 2-3--continued.

```
2281                                                                2340
CGGAACTGTTAATCATTTAATTCAATTTGCCTCAAATGATCTGCAAAGCGAGATGAAACA
  G   T   V   N   H   L   I   Q   F   A   S   N   D   L   Q   S   E   M   K   H
2341                                                                2400
TTACAGAGTTGGCGGAACACCAACACAGCGCATTAAACTCGAGGAGCAGCTGATTAAAGG
  Y   R   V   G/  G   T   P   T   Q   R   I   K   L   E   E   Q   L   I   K   G
2401                                                                2460
AATTTTCAAACCAAAACTTATGATGCAGCTCCTGCATGATGACCCATACATATTATTGCT
  I   F   K   P   K   L   M   M   Q   L   L   H   D   D   P   Y   I   L   L   L
2461                                                                2520
TGGCATGATCTCACCCACCATTCTTGTACATATGTATAGGATGCGTCATTTTGAGCGGGG
  G   M   I   S   P   T   I   L   V   H   M   Y   R   M   R   H   F   E   R   G
2521                                                                2580
TATTGAGATATGGATTAAGAGGGATCATGAAATCGGAAAGATTTTCGTCATATTAGAGCA
  I   E   I   W   I   K   R   D   H   E   I   G   K   I   F   V   I   L   E   Q
2581                                                                2640
GCTCACACGCAAGGTTGCTCTGGCTGAAGTTCTTGTGGATCAACTTAACTTGATAAGTGA
  L   T   R   K   V   A   L   A   E   V   L   V   D   Q   L   N   L   I   S   E
2641                                                                2700
AGCTTCACCACATTTACTTGAAATTATGAAGGGTTGTCAAGATAATCAGAGGGCATACGT
  A   S   P   H   L   L   E   I   M   K   G   C   Q   D   N   Q   R   A   Y   V
2701                                                                2760
ACCTGCGCTGGATTTGTTAACGATACAAGTGGAGCGTGAGTTTTCAAATAAAGAACTCAA
  P   A   L   D   L   L   T   I   Q   V   E   R   E   F   S   N   K   E   L   K
```

Fig. 2-3--continued.

2761 2820

AACCAATGGTTATCCCGATTTGCAGCAAACGCTCTTCGATATGAGGGAAAAAATGTATGC

T N G Y P D L Q Q T L F D M R E K M Y A

2821 2880

AAAGCAGCTGCACAATTCATGGCAAGAGCTAAGCTTGCTGGAAAAATCCTGTGTAACCGT

K Q L H N S W Q E L S L L E K S C V T V

2881 2940

GCGATTGAAGCAATTCTCGATTTTTACGGAAAGAAATTTAATCCAGCGAGCAAAAGAAGG

R L K Q F S I F T E R N L I Q R A K E G

2941 3000

AAAGCGCGCATCTTCGCTACAATTTGTTCACGAGTGTTTTATCACGACCCGAGTACATGC

K R A S S L Q F V H E C F I T T R V H A

3001 3060

GAAGAGCATTCGCGATGCAGGCGTGCGCAAACTAAATGAGGCTCTCGTCGGAATTTGTAA

K S I R D A G V R K L N E A L V G I C K

3061 3120

ATTCTTTTTCTCTTGTGGTTTCAAAATTTTTGCACGATGCTATAGCGACATAATATACCT

F F F S C G F K I F A R C Y S D I I Y L

3121 3180

TGTGAACGTGTGTTTGGTTTTCTCCTTGCTGCTACAAATGTCCAATACTGTGCGCAGTAT

V N V C L V F S L L L Q M S N T V R S M

3181 3240

GATAGCAGCGACAAGGGAAGAAAAAGAGAGAGCGATGGCAAATAAAGCTGATGAAAATGA

I A A T R E E K E R A M A N K A D E N E

Fig. 2-3--continued.

3241 3300

AAGGACGTTAATGCATATGTACCACATTTTCAGCAAGAAACAGGATGATGCGCCCATATA

R T L M H M Y H I F S K K Q D D A P I Y

3301 3360

CAATGACTTTCTTGAACATGTGCGTAATGTGAGACCAGATCTTGAGGAAACTCTCTTGTA

N D F L E H V R N V R P D L E E T L L Y

3361 3420

CATGGCTGGCGTAGAAGTTGTTTCAACACAGGCTAAGTCAGCGGTTCAGATTCAATTCGA

M A G V E V V S T Q A K S A V Q I Q F E

3421 3480

GAAAATTATAGCTGTGTTGGCGCTGCTTACCATGTGCTTTGACGCCGAAAGAAGCGATGC

K I I A V L A L L T M C F D A E R S D A

3481 3540

CATTTTCAAGATTTTGACAAAACTCAAAACAGTTTTTGGTACGGTTGGAGAAACGGTCCG

I F K I L T K L K T V F G T V G E T V R

3541 3547

ACTTCAA

L Q /

Fig. 2-3--continued.

(TEV, Verchot et al., 1991) to be important for proteolytic processing between P1 and AI proteins.

In the N-terminus of the AI region, the consensus sequence for aphid transmission is reported to be Cys-Gly-Val-Ala-Ala----Pro-Cys-Lys-Ile-Tyr-Cys--Cys (Thornbury et al., 1990), where Lys is the putative amino acid required for aphid transmission. The corresponding sequence for ZYMV-FL/AT is Cys-Gly-Leu-Ala-Ala----Pro-Val-Lys-Leu-Ser-Cys--C (Fig. 2-3). This consensus sequence for ZYMV-FL/AT is the same as that for ZYMV-CA and ZYMV-RU (Baker et al., 1991b). The two amino acid residues required for protease activity of the HC/Pro (Oh and Carrington, 1989) are also conserved with a Cys at position 652 and a His at position 725 (Fig. 2.3).

The deduced amino acid sequence of P1 of ZYMV-FL/AT was used to prepare a hydrophobicity plot according to Kyte & Doolittle (1982) (Fig. 2-4). The P1 protein is highly hydrophilic except for a strongly hydrophobic N-terminal 20 amino acids. There is no obvious pattern to suggest a transmembrane motif. The P1 of ZYMV-FL/AT also has a high proportion of basic amino acids (Fig. 2-5) and is thus highly positively charged. In contrast, the hydrophobicity map for P3 (Fig. 2-6) shows a distribution of both hydrophobic and hydrophilic regions, and a fairly random distribution of acidic as well as basic amino acids (Fig. 2-7). Interestingly, this plot is quite similar to that of

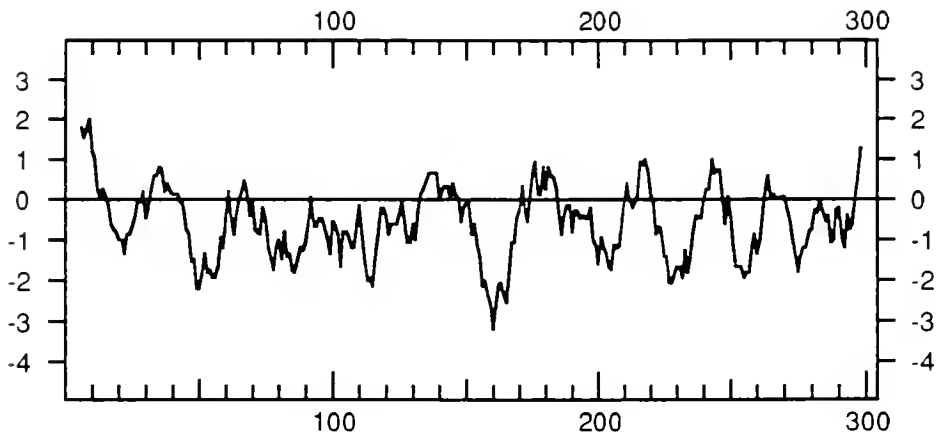

Fig. 2-4. Hydrophobicity of P1 of ZYMV-FL/AT plotted according to Kyte and Doolittle (1982). Horizontal scale represents amino acid residues of P1. Vertical scale represents hydrophobic amino acids (above the midline) and hydrophilic amino acids (below the midline).

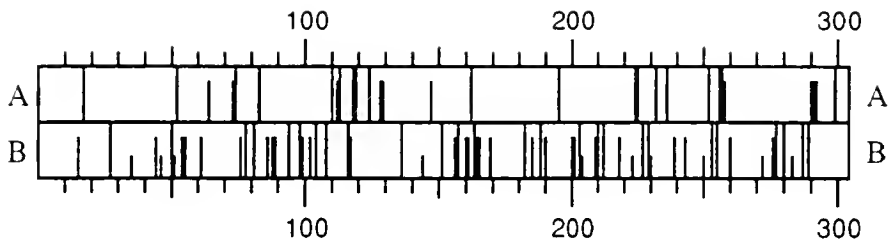

Fig. 2-5. Map of acidic (A) and basic (B) residues of the P1 protein of ZYMV-FL/AT.

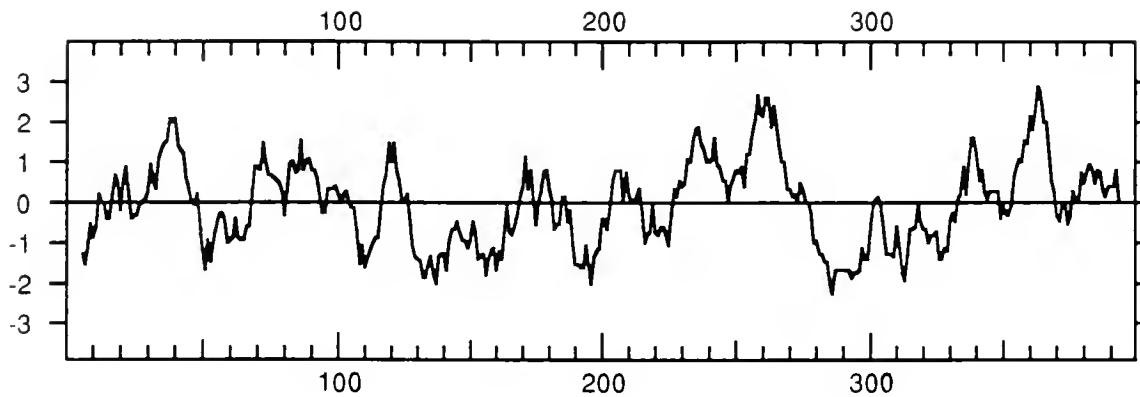

Fig. 2-6. Hydrophobicity plot of the P3 sequence of ZYMV-FL/AT. The horizontal scale indicates amino acid residues in the P3 protein. The hydrophobicity (vertical) scale is that of Kyte and Doolittle (1982), with hydrophobic amino acids above the midline and hydrophilic amino acids below the midline. Residues for ca. 35-50 and ca. 260-280 correspond to those suggested for P3 of TVMV to be involved in the formation of membrane spanning helices (Rodriguez-Cerezo and Shaw, 1991).

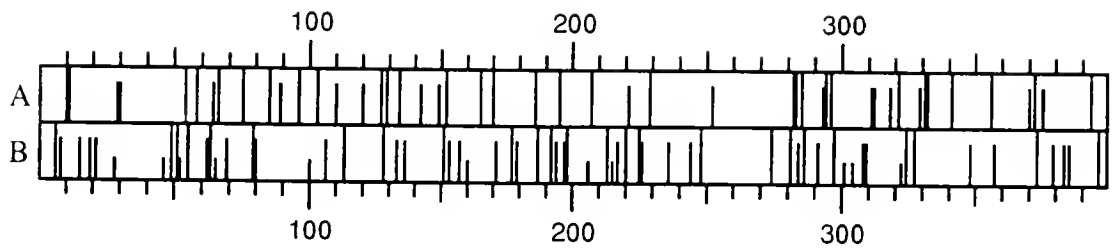

Fig. 2-7. Map of acidic (A) and basic (B) amino acid residues of the P3 protein of ZYMV-FL/AT.

the P3 of TVMV (Rodriguez-Cerezo and Shaw, 1991). Two areas of the P3 of ZYMV-FL/AT, indicated in Fig. 2-6, corresponded to the possible membrane spanning helices suggested by Rodriguez-Cerezo and Shaw.

Homologies Between the P1 of ZYMV Isolates

Comparisons were made between the P1 encoding regions of five ZYMV isolates: ZYMV-CA, ZYMV-RU, ZYMV-FL/AT, ZYMV-SV, and ZYMV-MD. The latter three isolates were sequenced in the present study. The sequence of P1 from ZYMV-FL/AT was derived from clones produced in λgt11 and λZAPII. The sequences of P1 from ZYMV-SV and ZYMV-MD were derived from clones produced by RNA-PCR products using custom primers for P1. The specific primers for production of P1 were, on the 5′-terminus, 5′-CATGAGAATTCAAGCTTACATGGCCTCTATCATG-3′, and on the 3′-terminus, 5′-CTGACTTCTAGACCTGTTCCAGCGCGTTCA-3′. Initial comparison of P1 products from RNA-PCR in agarose gels between the mild and severe isolates showed a size difference between the two, with the mild being slightly larger (Fig. 2-8). Nucleotide sequence comparisons showed an 18 nt insert in the mild isolate immediately after the start codon (Fig. 2-9), thus accounting for the size difference noted in agarose gels. The sequences between the ZYMV-FL/AT, ZYMV-SV and ZYMV-MD were quite similar, with a nt homology of 98% between ZYMV-FL/AT and the ZYMV-SV isolate, and a 95% homology between the ZYMV-FL/AT and the ZYMV-MD isolate. Of ten amino acid changes in the ZYMV-SV

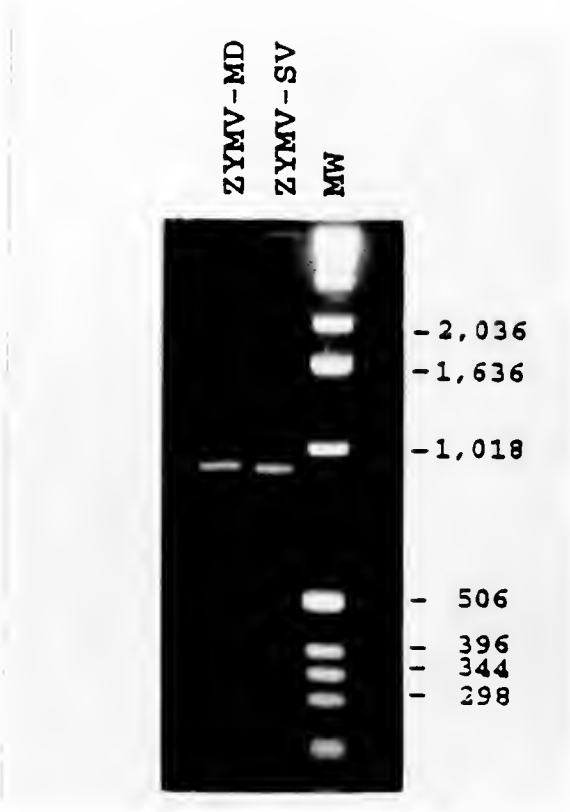

Fig. 2-8. Electrophoretic analysis of PCR products of P1 from ZYMV-MD and ZYMV-SV using primers specific to the P1 coding region in a 0.9% agarose gel.

Fig. 2-9. Nucleotide sequence alignments of the P1 coding region from ZYMV-FL/AT with that of ZYMV-CA, -SV, -MD, and -RU. Clones of ZYMV-SV and ZYMV-MD extend only to the GGAACAGG motif as shown due to the primer selection for PCR cloning.

```
    1                                                            50
FL ATG....... .......... .GCCTCTATC ATGATTGGTT CAATCTCCGT
CA ATG....... .......... .GCCTCCATC ATGATTGGTT CAATCTCTGT
SV ATG....... .......... .GCCTCTATC ATGATTGGTT CAATCTCGGT
MD ATGAGAATTC AAGCTTTACA TGCCTCTATC ATGATTGGTT CAATCTCTGT
RU ATG....... .......... .GCCGCTATC ATGATTGGTT CAATCTCTGT

    51                                                          100
FL ACCCATTGCA AAGACTAAGC AGTGTGCAAA CACTCAAGTA AGTAATCGGG
CA ACCCATTGCA AAGACTGAGC AGTGTGCAAA CACTCAAGTA AGTAATCGGG
SV ACCCATTGCA AAGACTGAGC AGTGTGCAAA CACTCAAGTA AGTAATCGGG
MD ACCCATTGCA AAGACTGAGC ACTGTGCAAA CACTCAAGTA AGTAATCGGG
RU CCCTATCGTT GAGTCTGCTC GGTGTGCAAC GGTTCAAACT GGAAACCGTG

   101                                                          150
FL TTAATATAGT GGCACCTGGC CACATGGCAA CATGCCCATT GCCACTGAAA
CA CTAATATAGT GGCACCTGGC CACATGGCAA CATGCCCATT GCCACTGAAA
SV TTAGTATAGT GCCACCTGG. ..CATGGCAA CATGCCCATT GCCACTGAAA
MD TTAATATAGT GGCACCTGGC CACATGGCAA TATGCCCATT GCCACTGAAA
RU TGAATATTGT GGCACCTGGC CACGTGGCAG TTTGCAAGCC ACAAATGAAA

   151                                                          200
FL ACGCACATGT ATTACAGGCA TGAGTCCAAG AA...GTTGA TGCAATCAAA
CA ACGCACATGT ATTACAGGCA TGAGTCCAAG AA...GTTGA TGCAATCAAA
SV ACGCACATGT ATTACAGGCA TGAGTCCAAG AA...GTTGA TGCAATCAAA
MD ACGCACATGT ATTACAGGCA TGAGTCCAAG AA...GTTGA TGCAATCAAA
RU TCGCACTCAT ATTACAAACA TGCATCAGAG AAACTCTCCA AACAAGCTAG

   201                                                          250
FL CAAAAGCATT GACATTCTGA ACAATTTCTT CAGCACTGA. ..CGAGATGA
CA CAAGAGCATT GACATTCTGA ACAACTTCTT CAGCACTGA. ..CGAGATGA
SV CAAAAGCATT GACATTCTGA ACAATTTCTT CAGCACTGA. ..CGAGATGA
MD CAAAAGTATT GACATTCTGA ACAATTTCTT CAGCACTGA. ..CGAGATGA
RU TGAAAGCATT AATATCCTCA ATAGTTTCTT TGACACTGAT CCAGAGATGC

   251                                                          300
FL AGTTTAGGCT CACTCGAAAC GAGATGAGCA AGGTGAAAAA GGGTCCGAGT
CA AGTTTAGGCT CACTCGAAAC GAGATGAGCA AGCTGAAAAA GGGTCCGAGC
SV AGTTTAGGCT CACTCGAAAC GAGATGAGCA AGGTGAAAAA GGGTCCGAGT
MD AGTTTAGTGT CACTCGAAAC GAGATGAGCA AGGTTAAAAA GGGTCCGAGT
RU GTTTTAGGCT CACTCGCAAT GAGATGAGCA AGGTAAAGAA GGGGCCAAAT

   301                                                          350
FL GGGAGGATAG TCCTCCGCAA GCCGAGTAAG CAGCGGGTTT TCGCTCGTAT
CA GGGAGGATAG TCCTCCGCAA GCCGAGTAAG CAGCGGGTTT TCGCTCGTAT
SV GGGAGGATAG TCCTCCGCAA GCCGAGTAAG CAGAGGGTTT TCGCTCGTAT
MD GGGAGGATAG CCCTCCGCAA GCCGAGTAAG CAGCGGGTTT TCGCTCGTAT
RU GGAAGGATGA TACTCCGCAA ACCAAGAGCA CAACGTGTTT TGGAGCGTAT
```

```
   351                                                         400
FL TGAGCAGGAT GAGGCAGCAC GCAAGAAAGA GACTGTTTTC CTCGAAGGAA
CA CGAGCAGGAT GAGGCAGCAC GCAAGGAAGA GGCTGTTTTC CTCGAAGGAA
SV TGAGCAGGAT GAGGCAGCAC GCAAGGAAGA GGCTGTTTTC CTCGAAGGAA
MD TGAGCAGGAT GAGGCAGCAC GCAAGGAAGA GGCTGTTTTC CTCGAAGGAA
RU CAGCTTTGAA AAGATCGAAA AAGGAGCAGA AAGACAAGTT CTACCATGGC

   401                                                         450
FL ATTATGACGA TTCTATCACA AATCTAGCAC GTGTTCTTCC ACCTGAAGTG
CA ATTATGACGA TTCCATCACA AATCTAGCAC GTGTTCTTCC ACCTGCCGTG
SV ATTATGACGA TTCGATCATA AGTCTAGCAC GTGTTCTTCC ACCTGAAGTG
MD ATTATGACGA TTCGATCACA AATCTAGCAC GTGTTCTTCC ACCTGAAGTG
RU GAGTATATGC TACTGTGACG TCCATCATTA ATACATTCAC AGATGAAAGG

   451                                                         500
FL ACTCACAACG TTGATGTGAG CTTGACGTCA TCGTTTTACA AGCGCACATA
CA ACTCACAACG TTGATGTGAG CTTGCGATCA CCGTTTTACA AGCGCACATA
SV ACTCACAACG TTGATGTGAG CTTGACGTCA CCGTTTTACA AGCGCACATA
MD ACTCACAACG TTGATGTGAG CTTGACGTCA CCATTTTACA AGCGCACATA
RU AATGGCATAG CTAACTCAAG TTTGCGCTCA CCGTTCTATA AACGTTCATG

   501                                                         550
FL CAAGAAGGAA AGGAAGAAAG TGGCGCAAAA GCAAATTGTG C...AAGCAC
CA CAAGAAGGAA AGGAAGAAAG TGGCGCAAAA GCAAATTGTG C...AAGCAC
SV CAAGAAGGAA AGGAAGAAAG TGGCGCAAAA GCAAATTGTG C...AAGCAC
MD CAAGAAGGAA AGGAAGAAAG TGGCGCAAAA GCAGATTGTG C...AAGCAC
RU CAGAAAGGAA AAGAAGAAAA TAGTATGTGA AAATGTTGTG CGTTCAGCCA

   551                                                         600
FL CACTCAATAG CTTGTGCACA CGTGTTCTTA AAATTGCACG CAATAAAAAT
CA CACTTAATAG CTTGTGCACA CGTGTTCTTA AAATTGCACG CAATAAAAAT
SV CACTTAATAG TTTGTGCACA CGTGTTCTTA AAATTGCACG CAATAAAAAT
MD CACTTAATAG CTTGTGCACA CGTGTTCTTA AAATTGCACG CAATAAAAAT
RU GTGTTAATAA TCTGTGCGAT CGCGTTCTCA AGATAGCGCG GGAGAAAAAC

    601                                                        650
FL ATCCCTGTTG AGATGATTGG CAACAAGAAG GCGAGACATA CACTCACCTT
CA ATCCCTGTTG AGATGATTGG CAACAAGAAG ACGAGACATA CACTCACCTT
SV ATCCCTGTTG AGATGATTGG CAACAAGAAG GCGAGACATA CACTCACCTT
MD ATCCCTGTTG AGATGATTGG CAACAAGAAG GCGAGACATA CACTCACCTT
RU ATTCCAGTTG AAATGATTGG AAAGAAAAAG AATCGACACA CCCTCACCTT

   651                                                         700
FL CAAGAGGTTT AGGGGATATT TTGTTGGAAA GGTGTCAGTT GCGCATGAAG
CA CAAGAGGTTT AGGGGATGTT TTGTTGGAAA GGTGTCAGTT GCGCATGAAG
SV CAAGAGGTTT AGGGGATGTT TTGTTGGAAA GGTGTCAGTT GCGCATGAAG
MD CAAGAGGTTT AGGGGATATT TTGTTGGAAA GGTGTCAGTT GCGCATGAAG
RU CAAGAACTTT AAGGGATCTT TCATTGGGAA AGTTTCATTA GCACACGAAA
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Fig. 2-9--continued.

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   701                                                        750
FL AAGGACGAAT GCGGCGCACT GAGATGTCGT ATGAGCAGTT TAAATGGATT
CA AAGGACGAAT GCGGCACACT GAGATGTCGT ATGAGCAGTT TAAATGGCTT
SV AAGGACGAAT GCGGCGCACT GAGATGTCGT ATGAGCAGTT TAAATGGATT
MD AAGGACGAAT GCGGCGCACT GAGATGTCGT ATGAGCAGTT TAAATGGATT
RU GGGGCCAAAT GAGACATGTT GAGATGTCGT ACGAACAGTT TGGATTCATT

   751                                                        800
FL CTAAAAGCCA TTTGTCAGGT CACCCATACA GAGCGAATTC GTGAGGAAGA
CA CTTAAAGCCA TTTGTCAGGT CACCCATACA GAGCGAATTC GTGAGGAAGA
SV CTAAAACCCA TTTGTCAGGT CACCTATACA GAGCGAATTC GCGAGGAAGA
MD CTAAAAGCCA TTTGTCAGGT CACCCATACA GAGCGAATTC GTGAGGAAGA
RU CTACAAGCCA TCTGTCGGGT TACGAACACA AGATGTGTGC GCGATGAGGA

   801                                                        850
FL TATTAAACCA GGTTGTAGTG GGTGGGTGTT GGGCACTAAT CATACATTGA
CA TATTAAACCA GGTTGTAGTG GGTGGGTGTT GGGCACTAAT CATACATTGA
SV TATTAAACCA GGTTGTAGTG GGTGGGTGTT GGGCACTAAT CATACATTGA
MD TATTAAACCA GGTTGTAGTG GGTGGGTGTT GGGCACTAAT CATACATTGA
RU CATCAAGCCG GGGTGTAGCG GATGGGTTCT AGGCGATGAT CACGAACTTA

   851                                                        900
FL CTAAAAGATA TTCAAGATTG CCACATTTGG TGATTCGAGG TAGAGACGAC
CA CTAAAAGATA TTCAAGATTG CCACATTTGG TGATTCGAGG TAGAGACGAC
SV CTAAAAGATA TTCAAGATTG CCACATTTGG TGATTCGAGG TAGAGATGAC
MD CTAAAAGATA TTCAAGATTG CCACATTTGG TGATTCGAGG TAGAGACGAC
RU CTCAGAAATT TTCGAGGTTA CCATGCCTAG TAATTCGTGG TAGAGATGAT

   901                                                942
FL GATGGGATTG TGAACGCGCT GGAACAGGTG TTATTTTATA GC
CA GATGGGATTG TGAACGCGCT GGAACAGGTG TTATTTTATA GC
SV GATGGGATTG TGAACGCGCT GGAACAGG.. .......... ..
MD GATGGGATTG TGAACGCGCT GGAACAGG.. .......... ..
RU GAAGGAATTG TGAATGCATT AGAACCAGTG TTCTTCTATG AT
```

Fig. 2-9--continued.

```
   1                                                          50
FL M------ASI MIGSISVPIA KTKQCANTQV SNRVNIVAPG HMATCPLPLK
MD .RIQALH... .......... ..EH...... .......... ...I......
RU .-------.A. .........V ESAR..TV.T G......... .V.V.KPQM.
SV .------... .......... ..E....... ....S..P.. -.........
CA .------... .......... ..E....... ...A...... ..........
   51                                                         100
FL THMYYRHESK KLMQSNK-SI DILNNFFSTD -EMKFRLTRN EMSKVKKGPS
MD .......... .......-.. .......... -....SV... ..........
RU S.S..K.A.E ..SKQASE.. N...S..D.. P..R...... .........N
SV .......... .......-.. .......... -......... ..........
CA .......... .......-.. .......... -......... ....L.....
   101                                                        150
FL GRIVLRKPSK QRVFARIEQD --E-AARKKE TVFLEGNYDD SITNLARVLP
MD ...A...... .......... --.-....E. A......... ..........
RU ..MIL...RA ...LE..SFE KI.KG.ERQV LPWRVYATVT ..I.TFTDER
SV .......... .......... --.-....E. A......... ..IS......
CA .......... .......... --.-....E. A......... ..........
   151                                                        200
FL PEVTHNVDVS LTSSFYKRTY KKERKKVAQK QIVQAP-LNS LCTRVLKIAR
MD .......... ...P...... .......... ......-... ..........
RU NGIA-NS--. .R.P....SC R..K..IVCE NV.RSASV.N ..D.......
SV .......... ...P...... .......... ......-... ..........
CA .A........ .R.P...... .......... ......-... ..........
   201                                    *                  250
FL NKNIPVEMIG NKKARHTLTF KRFRGYFVGK VSVAHEEGRM RRTEMSYEQF
MD .......... .......... .......... .......... ..........
RU E......... K..N...... .N.K.S.I.. ..L...R.Q. .HV.......
SV .......... .......... .....C.... .......... ..........
CA .......... ...T...... .....C.... .......... .H........
   251                          *                             300
FL KWILKAICQV THTERIREED IKPGCSGWVL GTNHTLTKRY SRLPHLVIRG
MD .......... .......... .......... .......... ..........
RU GF..Q...R. .N.RCV.D.. .......... .DD.E..QKF ..PC-.....
SV .....P.... .Y........ .......... .......... ..........
CA .......... .......... .......... .......... ..........
   301
FL RDDDGIVNAL EQVLFYS
MD .......... ..
RU ...E...... .P.F..D
SV .......... ..
CA RDDDGIVNAL EQVLFYS
```

Fig. 2-10. Amino acid sequence alignment of the P1 proteins of five ZYMV isolates: ZYMV-FL/AT, -MD, -RU, -SV, and -CA. Conserved residues among ZYMV isolates are underlined. Asterisks indicate strictly conserved residues among potyviruses. ZYMV-MD and -SV terminate at -EQ due to primer selection for PCR.

isolate compared to ZYMV-FL/AT, five made a difference in the polarity or charge, and five changes made no difference. In addition there was a deletion of a His at position 41 in the ZYMV-SV isolate. The P1 from the ZYMV-MD isolate had an insert of 6 additional amino acids immediately following the initiation codon, compared to ZYMV-FL/AT. There were nine different amino acids in the N-terminal half of ZYMV-MD, seven of which made a difference in the charge or polarity.

The P1 of ZYMV-CA had a high degree of similarity compared to ZYMV-FL/AT (96%), whereas ZYMV-RU was highly divergent with only a 60% nt sequence homology compared to the P1 of ZYMV-FL/AT (Table 2-3). In spite of the variability seen among five P1 regions, certain consensus regions and amino acids believed to be involved or required for protease activity of P1 were conserved. For example, all five isolates had the amino acid consensus Gly-Xaa-Ser-Gly, the His at position 223 and Ser at position 264 (Fig. 2-10). The conserved potyvirus sequence of Phe-Ile-Val-Arg-Gly (Verchot et al., 1991) close to the P1/AI cleavage site, was slightly different, with a sequence of Leu-Val-Ile-Arg-Gly for all five isolates (Fig. 2-10).

Homologies Between P1, AI, and P3 of ZYMV Isolates and Other Potyviruses

Nucleotide and amino acid sequence comparisons were made between the P1, AI, and P3 of ZYMV-FL/AT with ZYMV-CA,

Table 2-3. Percentage of nucleotide and amino acid sequence homologies of the P1, amorphous inclusion (AI), and P3 regions of four ZYMV isolates and five distantly related potyviruses with respect to ZYMV-FL/AT.

Virus/isolate	P1	AI	P3
ZYMV-CA	96(97)[a]	98(98)	98(99)
ZYMV-SV	98(97)	nd[b]	nd
ZYMV-MD	95(97)	nd	nd
ZYMV-RU	60(70)	88 (96)	84 (96)
TEV	42(45)	52 (63)	43 (54)
PVY^N	37(46)	51 (64)	44 (49)
TVMV	39(48)	52 (64)	44 (52)
PPV	41(38)	52 (65)	45 (50)
PSbMV	39(37)	50 (58)	45 (52)

[a] Homologies were determined using the GAP alignment of the University of Wisconsin Genetics Computer Group Program. GAP calculates alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Nucleotide sequence homologies are listed first with amino acid homologies in ().

[b] nd=not determined

ZYMV-RU, TEV (Allison et al., 1986), potato virus Y-strain N(PVY^N) (Robaglia et al., 1989), TVMV (Domier et al., 1986), plum pox virus (PPV) (Lain et al., 1990), and pea seed-borne mosaic virus (PSbMV) (Johansen et al., 1991) (Table 2-3). Sequence comparisons also were made between ZYMV-FL/AT, and ZYMV-SV, and ZYMV-MD for P1.

The P1 of ZYMV-CA, ZYMV-SV, and ZYMV-MD had high nt and amino acid sequence homologies to ZYMV-FL/AT ranging from 96% (CA) to 98% (SV). However, the P1 gene of ZYMV-RU had a 60% nt and a 70% amino acid homology compared to the P1 of ZYMV-FL/AT, and this was the greatest difference seen between a single gene among five ZYMV isolates studied to date. These nt homology values are also compared to the P1 of TEV (42%), PVY^N (37%), TVMV (39%), PPV (41%), and PSbMV (39%). The amino acid homologies were higher than the nt homologies for each virus in most cases (Table 2-3).

The AI nt coding region of ZYMV-FL/AT was also compared to that of ZYMV-CA (98% homology) and ZYMV-RU (88%). The deduced amino acid sequence for ZYMV-CA was the same as the nt homology compared to ZYMV-FL/AT. However, the amino acid homology for the AI of ZYMV-RU was 98%, indicating that many of the nt changes made no difference in the amino acid sequence. The AI sequence comparisons to unrelated potyviruses were from 50% homology for PSbMV to 52% for TEV, TVMV, and PPV.

The nt sequence of the P3 of ZYMV-FL/AT showed a 98% homology with the P3 of ZYMV-CA and 84% homology with ZYMV-RU. The ZYMV-FL/AT and distantly related potyviruses had nt homologies to ZYMV-FL/AT ranging from 43-45%. The amino acid sequence homologies of P3 compared to ZYMV-FL/AT were slightly higher than the nt homologies (Table 2-3). In all nt and amino acid comparisons, the P1 was the least conserved region whereas the HC/Pro was more highly conserved than either P1 or P3.

Discussion

Attempts to clone the entire 5′-terminus of the ZYMV genome necessitated the use of custom primers to force cloning specifically for the 5′-end. This resulted in a series of clones which represented the entire ORF of the ZYMV genome.

High nucleotide sequence similarities among the P1 of ZYMV-FL/AT, -SV, -MD, and -CA isolates were noted, but a significant divergence was seen with the P1 of the ZYMV-RU isolate. The larger size of the P1 from ZYMV-MD as seen in PCR analysis in agarose gels was verified by sequences which showed a six amino acid insert after the start codon. Whereas the first five amino acids were Met-Ala-Ser-Ile-Met for ZYMV-FL/AT, -SV, and -CA, and Met-Ala-Ala-Ile-Met for ZYMV-RU, the corresponding sequence for ZYMV-MD was Met-Arg-Ile-Glu-Ale-Leu-His-Ala-Ser-Ile-Met. All the differences

seen in the amino acid sequence for the ZYMV-MD were in the N-terminal half of P1. Eight of the 11 amino acid differences (which include one deletion) in the ZYMV-SV isolate were also in the N-terminal half of P1.

The conserved residues in the C-terminus of P1 have been maintained to some degree in the five ZYMV isolates studied. The His and Ser residues important for protease activity were present in all five isolates of ZYMV. However, the consensus sequence reported for the five potyviruses analyzed by Verchot et al. (1991) is slightly different. Instead of Phe-Ile-Val-Arg-Gly, the sequence for ZYMV isolates is Leu-Val-Ile-Arg-Gly. This sequence suggests a possible inversion of amino acids Val and Ile at this point. In spite of the lower conservation of ZYMV-RU, it is interesting to note that the conserved regions are the same among all ZYMV isolates, as well as the putative Tyr/Ser cleavage site between P1 and AI.

Among the five ZYMV isolates in this study, and five other potyviruses which have been completely sequenced (Verchot et al., 1991), the P1 is the most variable region on the potyviral genome. In addition, the N-terminus of P1 is more variable than its C-terminus.

Although the AI was not the primary focus of this research, the homologies of the potyviruses and three ZYMV isolates addressed in this study were compared. The AI-encoding region was more highly conserved than that of P1,

with a high homology (98%) noted between ZYMV-FL and ZYMV-CA, and a lower (88%) homology between ZYMV-FL/AT and ZYMV-RU. Other potyviruses had sequence homologies compared to ZYMV-FL/AT in the range (51-52%) expected for distinct potyviruses (Shukla et al., 1991).

The homologies seen for the P3-encoding region were similar to those seen for the AI-encoding region, with a high homology (98%) between ZYMV-FL/AT and -CA, and a lower homology (84%) between ZYMV-FL/AT and -RU isolates. The homologies of distinct potyviruses compared to ZYMV-FL/AT were lower (43-44%), as expected.

It is clear from the sequence analyses presented in this study, that there is variation in the P1 coding region among ZYMV isolates. There is also greater variation in the P1 region than in the AI or the P3 regions. The ZYMV-RU isolate appears to have a more highly diverged P1 nt sequence than the other ZYMV isolates addressed in this study. According to the criteria proposed by Shukla et al. (1991), ZYMV-RU does not fit the category of either a strain or a distinct potyvirus, regardless of whether the P1, AI, or P3 regions are considered. In addition, the CP region of ZYMV-RU is 88% similar to that of ZYMV-CA (Baker, et al., 1991b). Polyclonal antisera to the CP of ZYMV-FL/AT and ZYMV-RU cross react in reciprocal SDS-immunodiffusion tests with the formation of spurs (Baker, et al., 1991a). Several monoclonal antibodies (MAbs) produced to ZYMV-FL/AT

(Appendix 1) show a low affinity to ZYMV-RU. The symptomatology of ZYMV-RU on a range of susceptible hosts is very similar to classical ZYMV symptoms (Baker et al., 1991a; H.Lecoq, unpublished).

CHAPTER 3
SEROLOGICAL CHARACTERIZATION OF THE P1 PROTEIN OF ZUCCHINI YELLOW MOSAIC VIRUS FROM FLORIDA

Introduction

The nt sequences of the P1 and and P3 coding regions are less conserved than other coding regions on the potyviral genome (Shukla et al., 1991). Furthermore, the N-terminus of the P1 protein is less conserved than its C-terminus. For these reasons, it was hypothesized that the antigenic characterization of the P1 and P3 proteins would prove useful in distinguishing different isolates of potyviruses.

In order to evaluate the potential of antigenic properties of P1 and P3 for distinction of ZYMV isolates, the following experimental approach was attempted: cloning and sequencing of the P1 and P3 encoding regions as described in chapter 2, expression of the encoded proteins in *E. coli*, production of antisera to the expressed proteins, and development of serological detection methods. These procedures were successful for the P1 protein of ZYMV-FL/At and ZYMV-RU, and this chapter describes the antigenic detection and characterization of the P1 proteins of ZYMV isolates by western blots of extracts from infected plants

and by immunofluorescence in infected tissues. Although the P3 of ZYMV was cloned and sequenced, the toxicity of this protein in E. coli prevented expression of P3 and preparation of antisera, so that no serological studies were conducted with the P3 protein. During the course of this study, Rodriguez-Cerezo and Shaw (1991) used a similar approach to obtain antisera to P1 and P3 of TVMV. Although the P3 of TVMV appeared to be toxic to E. coli, as seen by cessation of cell growth, P3 was expressed in low levels. They detected the P1 and P3 proteins of TVMV serologically in extracts from infected plants and protoplasts. The P3 of TEV is likewise toxic to E. coli and cannot be expressed in its entirety (V. Doljas, personal comm.).

Materials and Methods

Culture of Virus Isolates

Table 3-1 lists the isolates of ZYMV used in this study, their source, and the original host from which they were isolated. Host plants were maintained in a growth room under a ca. 16 hr day length with an average temperature of 23 C or in a greenhouse. Isolates obtained from outside the state of Florida were kept under quarantine conditions in a locked growth room. Two ZYMV isolates from Israel were kindly provided by Y. Antignus (Volcani Center, Bet Dagan, Israel). Three ZYMV isolates from France were kindly

Table 3-1. List of zucchini yellow mosaic virus isolates used for serological studies of the P1 protein.

Isolate	Origin	Host
FC-2000	FL-Alachua Co.	squash
FC-2050	FL-Dade Co.	squash
FC-2154	FL-Collier Co.	watermelon
ZYMV-RU	Reunion Island	*Momordica charantia*
Italy	Italy	zucchini
ZYMV-FL/AT	FL-Sumter Co.	zucchini
ZYMV-FL/GH[a]	FL-Sumter Co.	zucchini
ZYMV-SV	FL-Palm Beach Co.	squash
ZYMV-MD	FL-Alachua Co.	squash
81-25	FL-Sumter Co.	squash
FC-3182	FL-DeSoto Co.	zucchini
HAT	Israel	zucchini
NAT	Israel	zucchini
Egypt	ATCC 405	squash
Connecticut	ATCC 594	squash
Taiwan	ATCC 622	squash
PAT	France	muskmelon
weak	France	muskmelon
E15	France	muskmelon
FC-3179	FL-DeSoto Co.	zucchini
FC-3180	FL-DeSoto Co.	zucchini
FC-3181	FL-DeSoto Co.	zucchini

[a] ZYMV-FL/GH=an isolate of ZYMV maintained for several years by mechanical inoculation.

provided by H. Lecoq (INRA, Station de Pathologie Vegetale, Montfavet, France). Three additional isolates were obtained from the American Type Culture Collection (ATCC). The 81-25 culture was isolated by W.C. Adlerz and was obtained from D.E. Purcifull. All the remaining Florida isolates were from the collection of D.E. Purcifull and G.W. Simone. Host plants used for routine assay and maintenance were pumpkin (Cucurbita pepo L. 'Small Sugar'). Squash (C. pepo L. 'Early Prolific Straightneck'), watermelon [Citrullus lanatus (Thunb.) Matsumi & Nakai 'Crimson Sweet'], and cantaloupe (C. melo L. 'Hales Best Jumbo') were also used for some studies.

For mechanical inoculations, tissues were triturated in a mortar and pestle with 0.02 M potassium phosphate buffer, pH 7.5, with the addition of 600 mesh carborundum. The slurry was rubbed onto fully expanded cotyledons with sterile cheesecloth pads, and plants were rinsed gently with water several minutes after inoculation. Mock inoculations were made using extracts from noninoculated plants.

Increase of P1 and P3 by PCR for Subcloning and Expression

Based on the nt sequence of P1 and P3 of ZYMV-FL/AT, primers were made which correspond to the beginning and end of each protein encoding region with special attention to areas with a high GC content. For cloning of P1 of ZYMV-FL/AT, restriction sites with five flanking bases on the 5'-end were incorporated into the primers to provide for

directional cloning into the pETh vector (McCarty et al., 1991) at HindIII and BglII sites on the polylinker. The pETh vector was selected as the expression vector in this study. It is a modification of the original pET vector developed by Studier et al. (1990) for high level expression of genes under the control of the T7 RNA polymerase from bacteriophage T7. In this system, if the protein product is not toxic to E. coli, host transcription cannot compete after induction of the T7 promoter and almost all transcription becomes due to the T7 RNA polymerase.

Primers used for increase of the P1 of ZYMV-FL/AT are presented in chapter 2. The P1 of ZYMV-RU was also increased by PCR for subcloning and expression. The primer for the 5′-end of the ZYMV-RU P1 gene was the same as for ZYMV-FL/AT P1, but for the 3′-end the primer was 5′-GGGCTCTAGATGGTTCTAATGCAT-3′, which included a BglII site and four flanking bases on the 5′-end. Primers for increase of P3 by PCR were, on the 5′-terminus, 5′-GGCGGAACACCAACA-3′ and on the 3′-terminus was 5′-CCAACCGTACCAAAA-3′. The primers for P3 did not include restriction sites, and the P3 gene was blunt-end ligated into the pETh vector.

The correct reading frames were selected for P1 and P3, based on the nt and deduced amino acid sequence, to be in frame with the ATG initiation codon of pETh. For P1 of both ZYMV-FL/AT and ZYMV-RU the plasmid providing the correct reading frame was pETh-3b, and for P3 it was pETh-3c. In

all cases digestion at the engineered restriction sites on the primers was unsuccessful (even after protease K digestion and phenol/chloroform extraction), so the PCR product was blunt-end ligated into the SmaI-digested, CIAP-treated plasmid.

An intermediate E. coli host, strain HB101, was transformed (Sambrook et al., 1989) for initial studies. Plasmid mini-preps were made from transformants to determine, by sequence data, the presence of the correct gene, its orientation, and verification of the correct reading frame for expression. Primers used for sequencing were the T7 promoter primer and the pBR322 EcoRI site clockwise primer, both obtained from the University of Florida DNA synthesis facility.

Induction and Expression of P1 and P3 Proteins

Plasmid cultures which were identified as P1 and P3 in the correct orientation and reading frame were used to transform the appropriate host for expression, E. coli strain BL21DE3pLysS. A single transformant colony grown on LB containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) was raised in 5 ml of M9 medium (Sambrook et al., 1989) with 0.4% glucose and 0.5% tryptone at 37 C with shaking to an $O.D._{600}$ of 0.6. Cultures were divided into 2.5 ml aliquots. One 2.5 ml aliquot was induced with 1 mM IPTG. Both induced and noninduced cultures were allowed to grow an additional 3-4 hr at 37 C. Cells were harvested by

centrifugation at 5,000 *g*, pellets were resuspended in one half the original volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and frozen at -20 C overnight. The viscosity of the cell lysate necessitated sonication for 5 sec to allow for pipetting. Ten μl of each sample was mixed with an equal volume of Laemmli dissociating solution (LDS) (Laemmli, 1970), the mixtures were boiled for 2 min, and were subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R-250 (BRL, Gaithersburg, MD) for detection of expressed proteins. In all cases, both induced and noninduced recombinant plasmid cultures were tested, as well as plasmid cultures containing no inserted gene.

Since P3 appeared to be toxic to E. coli in this system, as determined by lack of cell growth and protein expression following induction, an alternate induction procedure was attempted using the bacteriophage CE6 to infect pETh plasmids containing P3 in the E. coli host HMS174. The HMS174 has no T7 RNA polymerase and the enzyme is provided by infection with CE6 bacteriophage. This expression can be useful for expression of toxic gene products (Studier et al., 1990).

Antigen Preparation and Antibody Production

Since the P1 proteins expressed by both ZYMV-FL/AT and ZYMV-RU were insoluble, the proteins were partially

purified, after sonication, by three cycles of centrifugation of the precipitate at 10,000 *g* and washing with TE buffer, followed by preparative SDS-PAGE on a 3 mm gel. Protein bands were visualized by incubating in 0.2 M KCl for 7 min at 4 C. The protein bands were excised, washed three times in cold distilled water, frozen at -20 C, and eluted using a Bio-Rad Electroeluter at 10 mAmp/tube, with constant current for 5 hr. Polarity was reversed for 1 min, and the extracted proteins were dialyzed overnight against distilled water. Purity of the eluted protein was checked by analytical SDS-PAGE, after which the protein was lyophilized.

A New Zealand white rabbit (no. 1181) was immunized on day one with 2 mg of ZYMV-FL/AT P1 protein in 0.5 ml sterile distilled water which was emulsified with 0.5 ml of Freund's complete adjuvant. Injections were made using 0.25 ml of emulsion per site, with two intramuscular sites per hip. On day 14 and day 21, 1 mg protein was used with 0.5 ml water and 0.5 ml Freund's incomplete adjuvant. The same schedule was followed for rabbit no. 1186 which was immunized with the P1 protein of ZYMV-RU. Rabbits were bled on a weekly basis starting on day 28 for 2 months, with a 4 week interval before a booster injection on day 112 and subsequent bleeding.

Western Blotting Procedure

The western blotting procedure was conducted essentially as described by Towbin et al. (1979) using a Bio-Rad Mini-Protean II Electrophoresis Cell and Bio-Rad Trans-Blot Electrophoretic Transfer Cell. Ten per cent gels were used primarily, with occasional 8 and 15% gels being run for special purposes.

Young, symptomatic leaves of inoculated test plants were harvested between days 5 and 21 post-inoculation. Extracts for immunoblots were initially prepared by triturating leaf tissue in LDS (1:1, w:v), followed by boiling for 2 min. However, for adequate extraction of P1 from plant tissues, an alternate extraction buffer (ES buffer) (Rodriguez-Cerezo and Shaw, 1991) was used which gave improved results. The ES buffer consisted of 75 mM Tris-HCl, pH 6.1 containing 9 M urea, 7.5% 2-mercaptoethanol, and 4.5% SDS. One part plant tissue was triturated in a mortar and pestle with 2 parts of ES buffer. The triturate was squeezed through a single layer of moistened cheesecloth, boiled 2 min, and centrifuged at 5,000 *g* for 5 min. Centrifuged samples were stored at -20 C. Each isolate was tested from at least two different sources of tissue.

Nitrocellulose membranes (Bio-Rad Trans-Blot, 0.4μ) were rinsed three times with TBST after transfer, followed by incubation for 15 min at RT with 10 ml blocking solution

containing E. coli lysate at 1 mg/ml and extracts from noninfected plants. The noninfected plant extract was prepared by triturating leaf tissue in water (1:9, w:v) and straining through a single layer of moistened cheesecloth. The specific primary antibody was added at 1/1000 dilution. The procedure for the secondary antibody and development are as described in chapter 2 for immunoscreening. Reactions were allowed to develop at RT and were stopped by rinsing in deionized water.

In Vitro Translation and Immunoprecipitation

The wheat germ (WG) in vitro translation procedure was the same as described by Cline et al. (1985). Three μg of RNA from ZYMV-FL/AT in a 50 μl WG extract mixture, containing 40 μCi of [H^3]leucine was incubated at 25 C for 60 min. Immunoprecipitation analyses were performed as described by Dougherty and Hiebert (1980). Precipitated products were separated on a 10% SDS-PAGE and detected on dried gels by fluorography as described by Bonner and Lasky (1974). Antisera used for immunoprecipitation of in vitro translation products were to the P1 and CP of ZYMV, and to the AI of PRSV-W.

Production of Antisera to Synthetic Peptides

Synthetic peptides to the N-terminus of both the P1 and P3 proteins of ZYMV were prepared by the University of Florida protein synthesizing facility. The amino acid sequence of the peptide prepared to P1 was Met-Ala-Ser-Ile-

Met-Ile-Gly-Ser-Ile-Ser-Val-Pro and to P3 was Gly-Thr-Pro-Thr-Gln-Arg-Ile-Lys-Leu-Glu-Glu-Gln. Both free peptide and peptide conjugated to BSA were used as immunogens. Peptides were coupled to BSA according to Harlow and Lane (1988). One mg peptide and 1 mg BSA were each dissolved in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0. Two hundred µl of fresh 25% glutaraldehyde was added to 1 ml of the protein:peptide solution and stirred overnight at room temperature. This was followed by dialysis against deionized water three times over a 24 hr period. The material was lyophilized and stored at -20 C. Immunization protocols followed were as described for the expressed P1 protein from E. coli. Rabbit numbers for P3 peptide and the conjugated P3 peptide were 1167 and 1169, respectively. The rabbit numbers for the P1 peptide and the conjugated P1 peptide were 1168 and 1170, respectively.

Light Microscopy and Immunofluorescence Tests

Indirect immunofluorescence tests were conducted as described by Hiebert et al. (1984) with some modifications. Six µl of 10% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS), 27 µl of healthy plant extract (1/10 in PBS containing 1% ovalbumin), and 27 µl of antiserum were incubated together for 30 min prior to addition of epidermal strips from plant tissue. Epidermal strips were incubated in the antibody preparation in a 1.5 ml microfuge tube after vortexing for 10 sec to ensure complete exposure of the

tissue to the antibody solution. Tissue was incubated in the antibody solution on a shaker for 3-4 hr at RT in the dark. Rinsing between steps was done twice in 1 ml of TBST after vortexing for 10 sec and once for 1 hr in PBS while shaking at RT in the dark. Rhodamine-conjugated protein A (Sigma Chemical Co., St. Louis, MO) was used as a fluorescent probe. The rhodamine-conjugate was diluted 1 g/ml in PBS. Eight μl of the conjugate was mixed with 40 μl of 10% DMSO and 352 μl of PBS. After vortexing, rinsed tissue was incubated in this solution at RT for 3-4 hr in the dark while shaking. After a final rinse, tissue was mounted on microscope slides using Aqua-mount (Lerner Labs, New Haven, CT). 'Crimson Sweet' watermelon was used as the host for immunofluorescence tests. Tissue sections were photographed with epifluorescence optics using a Nikon Fluophot microscope with a G2A filter.

Results

Expression of P1 and P3 Coding Regions in E. coli

Initial efforts to clone P1 by cohesive end ligation at digested sites of PCR products were unsuccessful. Subsequently, PCR products of P1 and P3 were blunt-end ligated successfully into the pETh plasmid. Clones were sequenced to determine the correct orientation and reading

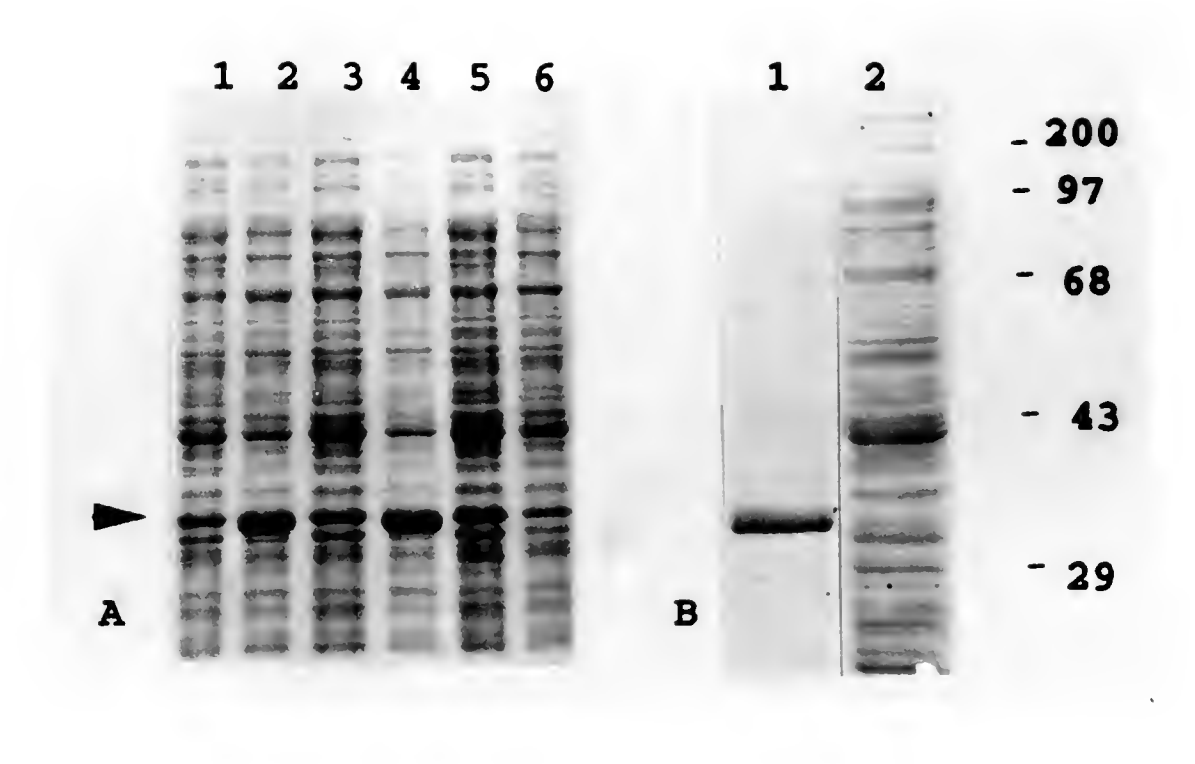

Fig. 3-1. Expression of P1 protein of ZYMV-FL/AT cloned into the pETh plasmid. A: Cultures expressing P1 were grown for 3 hrs after induction with IPTG. Noninduced cultures were processed the same as induced cultures. Lanes 2 and 4 are induced cultures carrying the P1 gene. Lanes 1 and 3 are noninduced cultures carrying the P1 gene. Lane 5 is a noninduced culture carrying the pETh plasmid only, and lane 6 is the same culture, induced. B: Lane 1 is the P1 protein which was partially purified by centrifugation prior to preparative electrophoresis, and lane 2 is the supernate from the same preparation. Arrow indicates the P1 expressed protein at ca. 35-kDa.

frame, and were then used to transform the appropriate E. coli host for expression, BL21DE3pLysS.

Induction of P1 from ZYMV-FL/AT resulted in overexpression of an insoluble protein product of ca. 36-kDa (Fig. 3-1A). Fifty ml cultures were induced for large scale P1 protein production. The P1 protein was insoluble and thus was easily purified by three cycles of centrifugation and washing, thereby providing a product free of mostbacterial proteins (Fig. 3-1B). Further purification of P1 protein from bacterial lysates was accomplished by preparative SDS-PAGE and electroelution. The protein product was then lyophilized and used for antiserum production in rabbits. Bleeding dates for P1 of ZYMV-FL/AT used were from 4 weeks to 7 months after the original series of immunizations. For the P1 of ZYMV-FL/AT, rabbit 1181 was bled from September 13, 1991 through July 16, 1992. The P1 of ZYMV-RU was expressed and purified in a similar manner for use as an immunogen. For the P1 of ZYVM-RU, rabbit 1186 was bled from May 28, 1992 through July 16, 1992.

Induction of the P3 protein was unsuccessful in both the pETh/BL21DE3pLysS system and when using the bacteriophage CE6 to infect the host HMS174 carrying the pETh plasmid (data not shown). These results are similar to the results with the TEV P3 protein (V. Doljas, personal comm.), which is toxic in E. coli and thus cannot be expressed in its entirety. Rodriguez-Cerezo and Shaw (1991)

expressed P3 of TVMV in E. coli. The induced bacterial cells ceased growth following induction with IPTG, and thus the level of expression was low (E. Rodriguez-Cerezo, personal comm.). Apparently the P3 protein of all three potyviruses is toxic to varying degrees in E. coli.

Detection of P1 Protein in Plants Infected with ZYMV

Initial extraction of ZYMV-infected plant tissues with LDS gave very weak reactions in western blots. Extraction with ES buffer gave satisfactory reactions with ZYMV-infected plant tissues in western blots using antiserum to P1 of ZYMV-FL/AT as a probe. The antiserum reacted specifically to a ca. 34-kDa protein in plant tissue infected with ZYMV-FL/AT. No protein was detected in extracts from healthy plant tissues. Western blots with preimmune serum did not result in a detectable protein reaction.

In western blots using antiserum to the P1 of ZYMV-FL/AT (1181), a P1 protein reaction at ca. 34-kDa for ZYMV-FL/AT was noted. Some heterogeneity was seen among the other ZYMV isolates used in this study (Fig. 3-2). A higher molecular weight (mw) of ca. 35-kDa was noted for some ZYMV isolates including ZYMV-MD, FC-2000, FC-2050, and three isolates from France (PAT, weak, and E15) (Table 3-2). P1 products that were slightly smaller than that of ZYMV-FL/AT included those of ZYMV-SV, three ATCC isolates of ZYMV (from Egypt, Taiwan, and Connecticut), and the original ZYMV

isolate from Italy. In addition to size differences of P1 protein, some isolates showed a possible breakdown product of ca. 26-27-kDa whereas others (FC-3182, weak and E15 from France) showed an incomplete processing of P1 and HC/Pro by the reaction of a band of ca. 88-kDa. This 88-kDa band of FC-3182 was tested and also reacted with antiserum to the AI of PRSV-W (Fig. 3-3).

The size heterogeneity between ZYMV-MD, ZYMV-FL/AT, and ZYMV-SV seen in SDS-PAGE using 10% acrylamide gels with antisera to the P1 of ZYMV-FL/AT as a probe, was further examined by subjecting them to SDS-PAGE in 8% and 15% acrylamide gels (Fig. 3-4). These size differences were also noted in the 8% and 15% gels, providing evidence that the heterogeneity seen is due to true mw differences between P1 proteins and not solely due to charge differences (Hedrick and Smith, 1968).

The size differences between the P1 proteins of ZYMV-FL/AT, ZYMV-MD, and ZYMV-SV were consistent regardless of the host used for western blot assays. These three isolates were tested in pumpkin, watermelon, cantaloupe, and squash (Fig. 3-5).

Of the ZYMV isolates tested in this study, ZYMV-RU reacted weakly or not at all with the antiserum to the P1 of ZYMV-FL/AT. This isolate did react with antisera to the CP and CI of ZYMV and to the AI of PRSV-W (Fig. 3-6). Extracts from pumpkin singly infected with any of several other

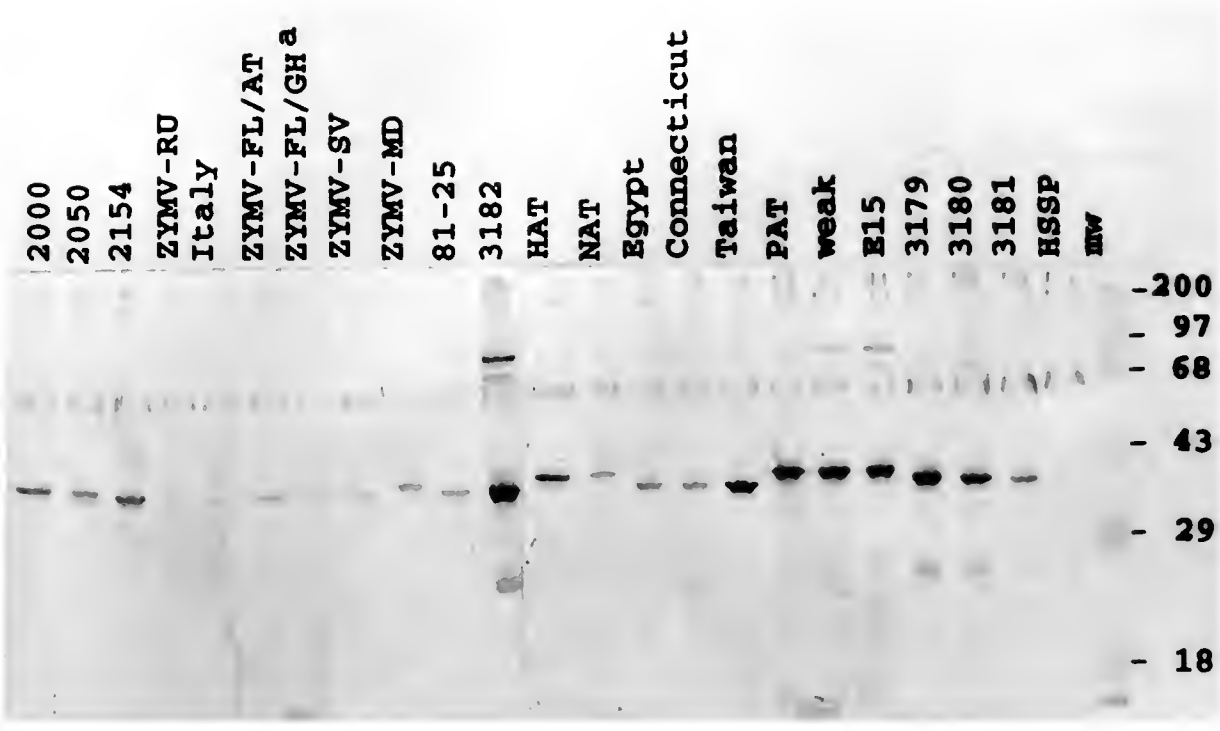

Fig. 3-2. Characterization of the reactions of the P1-related proteins of 22 isolates of zucchini yellow mosaic virus (ZYMV) in western blots. Blots were probed with antiserum no. 1181 (collection date 3-17-92) to the P1 protein of ZYMV-FL/AT.

Table 3-2. Evidence for antigenic and size variation of P1-related proteins among ZYMV isolates in western blots using antiserum to ZYMV-FL/AT (1181) and ZYMV-RU (1186).

Isolate	Approximate molecular weight	Reaction pattern(1181)	Reaction pattern(1186)
FC-2000	35 [a]	35	35
FC-2050	35	35	35
FC-2154	34	34,26	34
ZYMV-RU	33(+/-) [b]	33(+/-)	33
Italy	33	33	33(+/-)
ZYMV-FL/AT	34	34,26	- [c]
ZYMV-FL/GH	34	34,26	-
ZYMV-SV	33.5	33.5	-
ZYMV-MD	35	35	35
81-25	34	34	34
3182	34	88,34,26	88,34
HAT	34	34	34
NAT	34	34	34
Egypt	33	33	-
Connecticut	33	33	-
Taiwan	33	33	33
PAT	35	35,27	35
weak	35	88,35,27	35
E15	35	88,35,27	35
FC-3179	34	34,26	34
FC-3180	34	34,26	34
FC-3181	34	34	-

[a] molecular weights are in kDa.
[b] (+/-)=extremely weak reactions; not detectable in every test.
[c] - = no detectable reaction.

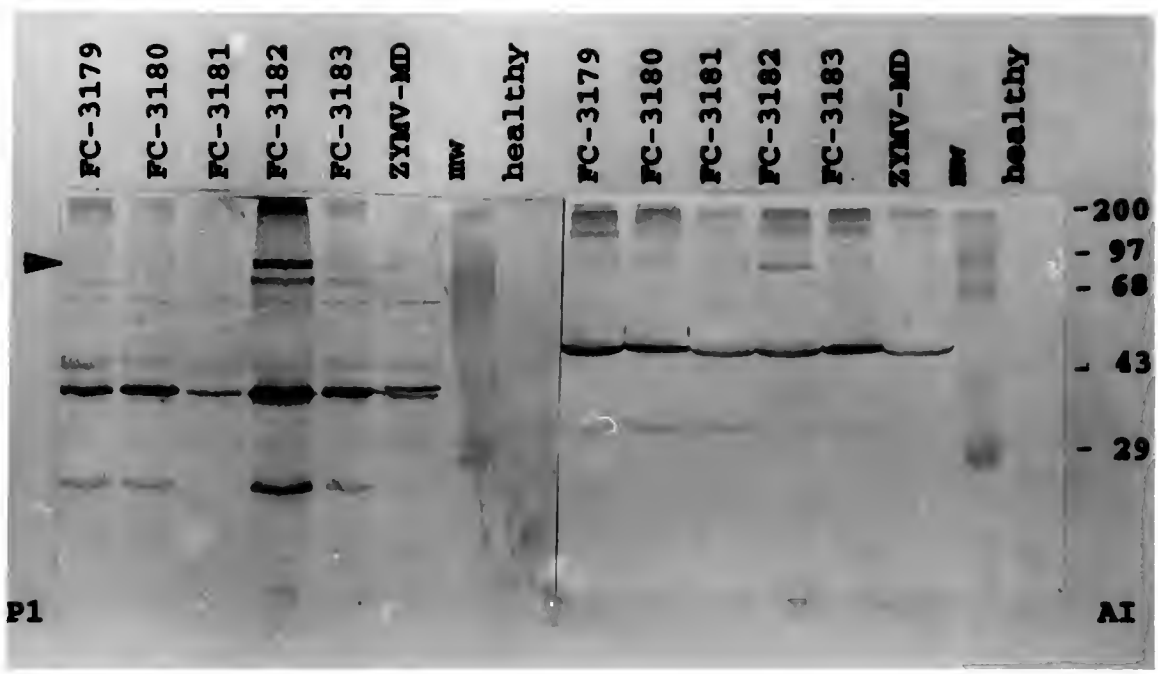

Fig. 3-3. Western blots of extracts from plants singly infected with selected ZYMV isolates. Blots were probed with antisera to P1 of ZYMV-FL/AT (P1) and to the AI of PRSV-W (AI). Arrow indicates the position of the 88-kDa protein of isolate FC-3182 which reacts with both P1 and AI antisera.

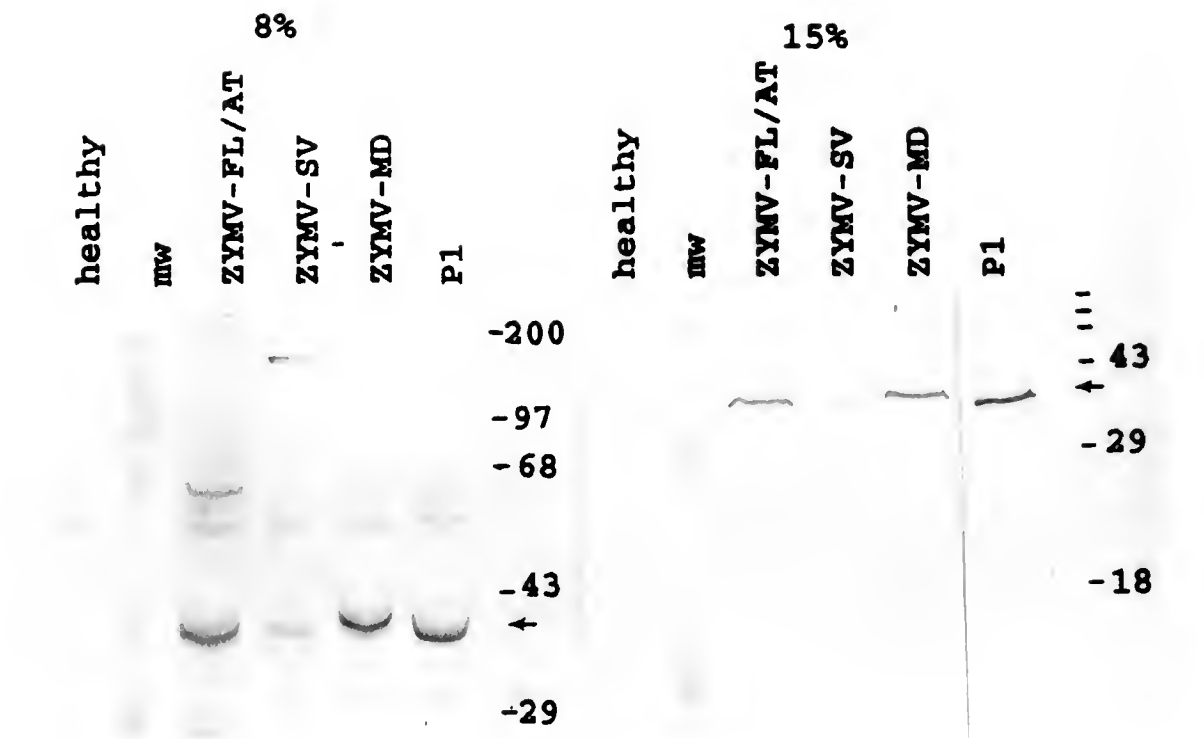

Fig. 3-4. Western blots showing 8% and 15% SDS-PAGE gel concentrations. Antiserum used to probe blots was to the P1 of ZYMFV-FL/AT. The size differences among the isolates shown were consistent regardless of the gel concentration. P1=protein expressed from E. coli. mw=molecular weight markers.

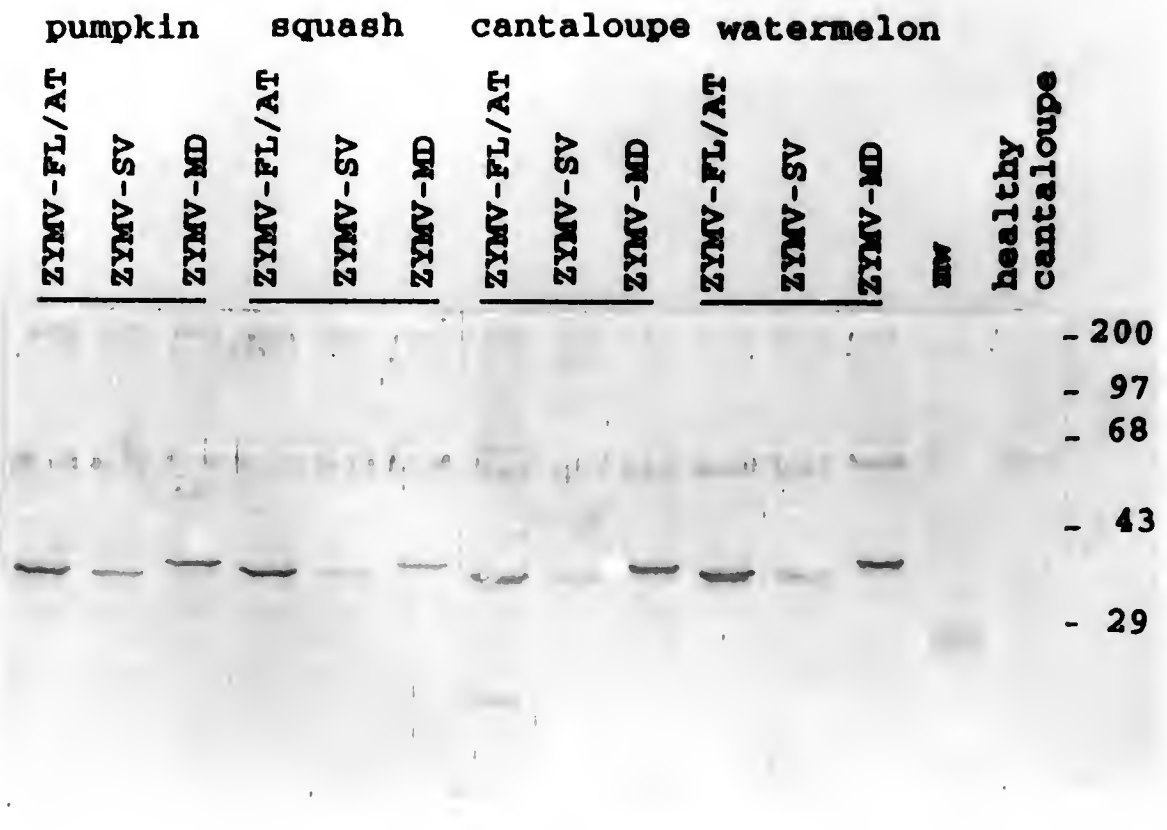

Fig. 3-5. Detection of the P1 protein in western blots of extracts from plants of four cucurbit cultivars singly infected with three isolates of zucchini yellow mosaic virus. The hosts were pumpkin (Cucurbita pepo 'Small Sugar'), squash (C. pepo 'Early Prolific Straightneck'), cantaloupe (Cucumis melo 'Hale's Best Jumbo'), and watermelon (Citrullus lanatus 'Crimson Sweet'). Antiserum no. 1181 (collection date 3-17-92) to P1 of ZYMV-FL/AT was used as the probe.

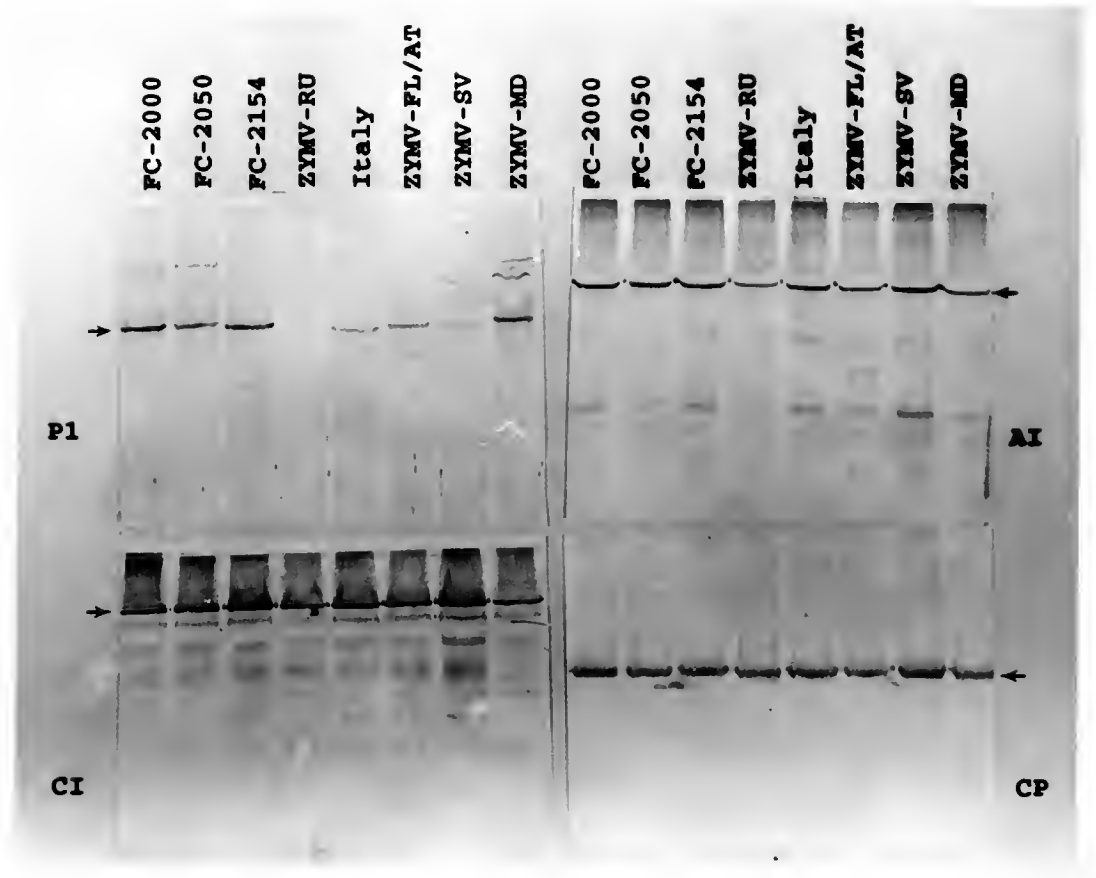

Fig. 3-6. Western blots of extracts from pumpkin singly infected with selected ZYMV isolates using antiserum to P1 of ZYMV-FL/AT, CI, and CP of ZYMV-FL/AT, and to the AI of PRSV-W as probes. The ZYMV-RU isolate reacted weakly or not at all with antiserum to the P1 of ZYMV-FL/AT, but reacts with other antisera. P1, membrane probed with antiserum to the P1 protein of ZYMV-FL/AT. AI, membrane probed with antiserum to the AI of PRSV-W. CI, membrane probed with antiserum to the CI of ZYMV-FL/AT. CP, membrane probed with antiserum to the CP of ZYMV-FL/AT. Arrangement of isolates is the same for all four membranes. Arrows indicate position of respective proteins in each membrane.

viruses that infect cucurbits but are distinct from ZYMV were also tested in western blots. These included PRSV-W, WMV-2, an unamed potyvirus (FC-2932) which is antigenically different from ZYMV, PRSV-W, and WMV-2 (Purcifull et al., 1991), cucumber mosaic virus (CMV), and a possible potexvirus of cucurbits (FC-1860, Purcifull et al., 1988). All of these extracts were negative in western blot tests when tested against antiserum to the P1 of ZYMV-FL/AT (Fig. 3-7). Antiserum to the P1 protein of ZYMV-RU was also negative when tested against PRSV-W, WMV-2, FC-1860, and FC-2932 (data not shown). This antiserum showed differential reactivity to several ZYMV isolates (Table 3-2, Fig. 3-8). For those isolates which reacted with antisera to both ZYMV-FL/AT P1 and ZYMV-RU P1, the approximate mw estimates for those isolates were the same.

Immunoprecipitation Analysis of In Vitro Translation Products

Translation products obtained in the WG in vitro translation system were immunoprecipitated with antisera to the P1 and CP of ZYMV-FL/AT, to the AI of PRSV-W and with preimmune serum. Since the WG translation system does not yield large products, analysis of SDS-PAGE showed only the P1 and AI present in total translation products (Fig. 3-9). The antisera to P1 and AI precipitated products of the appropriate mw for each, ca. 34-kDa and 52-kDa,

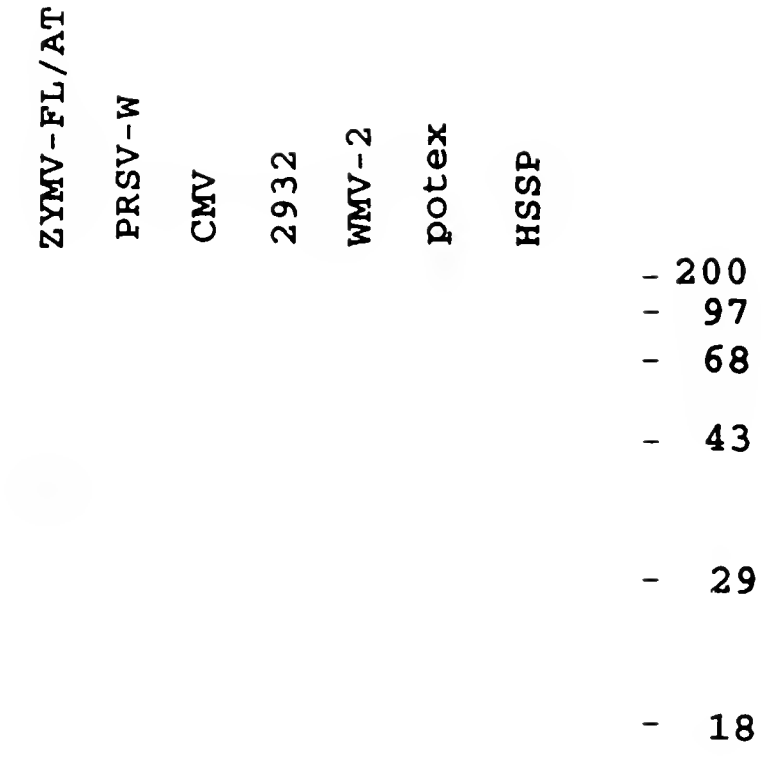

Fig. 3-7. Specificity of antiserum (no. 1181, collection date 3-17-92) to P1 of ZYMV-FL/AT in western blots. Extracts from samples infected with ZYMV-FL/AT show a prominent band at ca. 34-kDa and a weak band at ca. 26-kDa. Note lack of reactivity with extracts from pumpkin singly infected with any of three potyviruses (PRSV-W, WMV-2, 2932), a cucumovirus (CMV), a possible potexvirus (FC-1860, potex), or from noninoculated pumpkin leaves (HSSP/healthy).

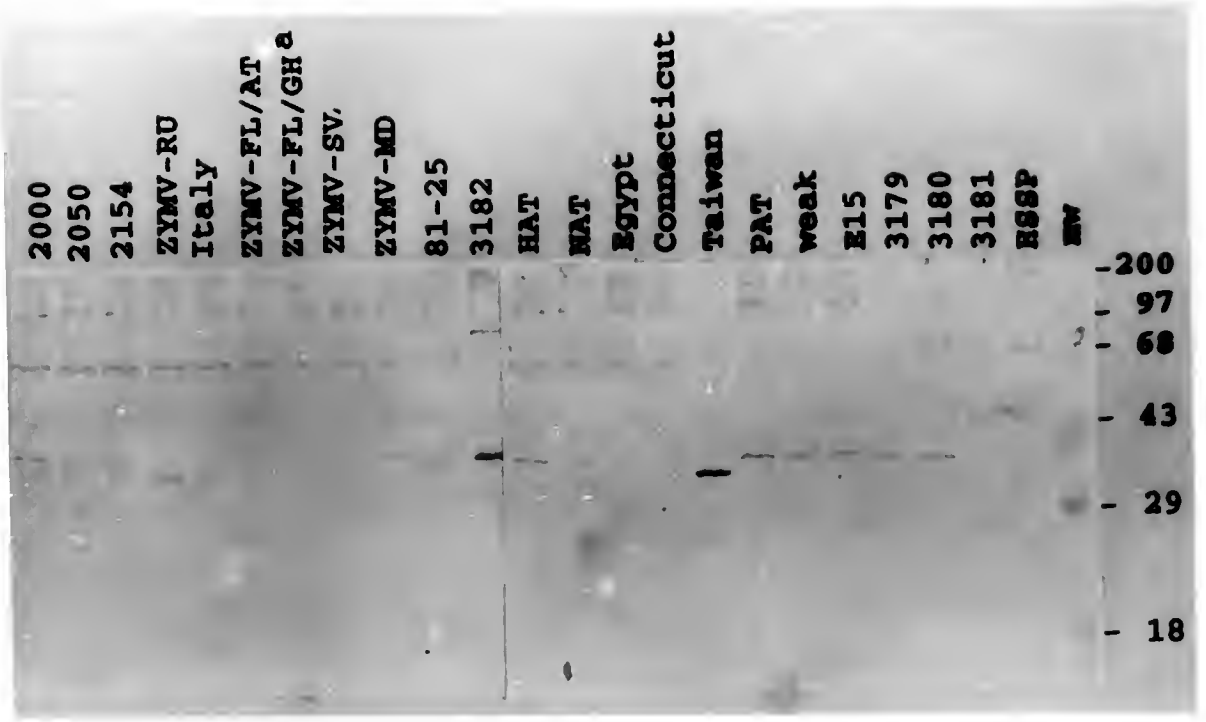

Fig. 3-8. Reactivity of 22 zucchini yellow mosaic virus (ZYMV) isolates in western blots using antiserum to the P1 of ZYMV-RU as a probe. Blots were probed with antiserum no. 1186 (collection date 6-10-92).

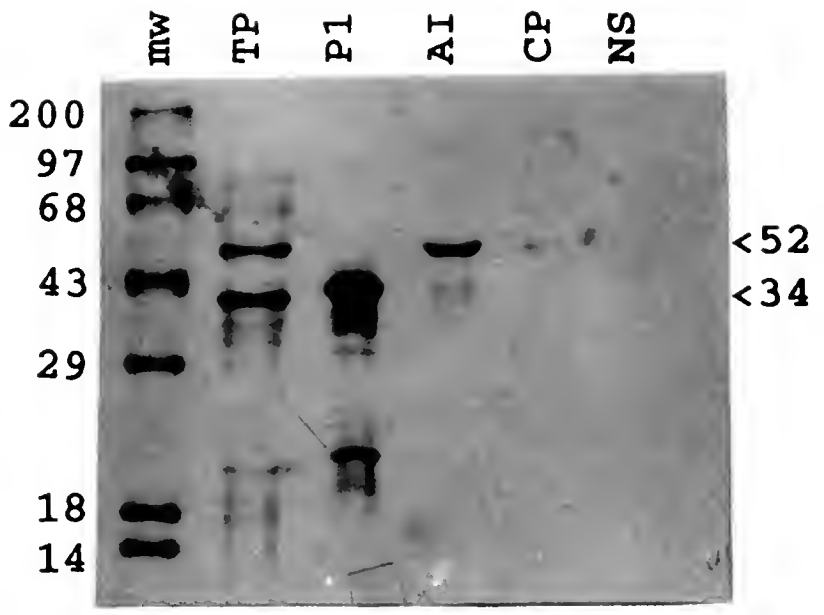

Fig. 3-9. Immunoprecipitation of wheat germ <u>in vitro</u> translation products. TP=total products, P1=products immunoprecipitated with ZYMV-FL/AT P1 antiserum, AI=products immunoprecipitated with antiserum to the AI of PRSV-W, CP=products immunoprecipitated with antiserum to the CP of ZYMV-FL/AT, and NS=products immunoprecipitated with preimmune serum. mw=molecular weights are from top to bottom: 200-, 97-, 68-, 43-, 29-, 18-, 14-kDa.

respectively. Neither the CP nor preimmune serum precipitated a protein product. Interestingly, the P1 antiserum precipitated a smaller product, ca. 25-kDa, which may be similar to the possible breakdown product usually seen in immunoblots for that isolate.

Detection of P1 Protein by Indirect Immunofluorescence

Fluorescence microscopy of rhodamine-conjugated protein A labeled antiserum to the P1 protein showed the presence of aggregates in the cytoplasm of epidermal strips from ZYMV-infected watermelon. Isolate ZYMV-FL/AT showed accumulation of amorphous aggregates with particulate fluorescing bodies in cells of epidermal tissues (Fig. 3-10) when treated with antiserum to the P1 of ZYMV-FL/AT. Similar results were seen with isolates ZYMV-SV and FC-3182. It is possible, judging from the location of the fluorescing material, that these bodies might be associated with the CI protein inclusions. Further studies using electron microscopy of ultrathin sections will be needed to ascertain this. Epidermal strips of ZYMV-FL/AT, ZYMV-SV and FC-3182 showed no fluorescence with preimmune serum as a probe (Fig. 3-11; Table 3-3), and the aggregates could be seen unstained in epidermal tissue. Likewise, tissues of watermelon infected with ZYMV-RU and healthy watermelon (mock-inoculated) were negative for fluorescence with both antiserum to the P1 of ZYMV-FL/AT and preimmune sera. As with the ZYMV-FL/AT, ZYMV-SV, and FC-3182, aggregates of P1 like those seen in

fluorescence tests could be seen in ZYMV-RU infected tissues, although the aggregates did not fluoresce (Fig. 3-12). Mock-inoculated watermelon tissues showed no fluorescence when stained with either immune (Fig. 3-13) or preimmune serum.

Tissues of ZYMV-FL/AT, ZYMV-SV, ZYMV-RU, FC-3182, and mock-inoculated watermelon were also tested in immunofluorescence studies using antiserum to the P1 of ZYMV-RU. Only watermelon tissue infected with ZYMV-RU or FC-3182 showed the particulate fluorescing aggregates, whereas ZYMV-FL/AT, -SV, and mock-inoculated tissues showed no fluorescing aggregates (Table 3-3). Although aggregates were seen in ZYMV-FL/AT and in ZYMV-SV infected tissues, they did not fluoresce.

Serological Studies of Synthetic Peptides to P1 and P3 of ZYMV

Antisera to the N-terminal 12 amino acids of P1 and P3 were used in SDS-immunodiffusion, ELISA, dot-immunoblots, and immunoblotting assays. None of the antisera, whether to the conjugated peptide or to the free peptide, reacted with any detectable protein product from ZYMV-infected plant tissues.

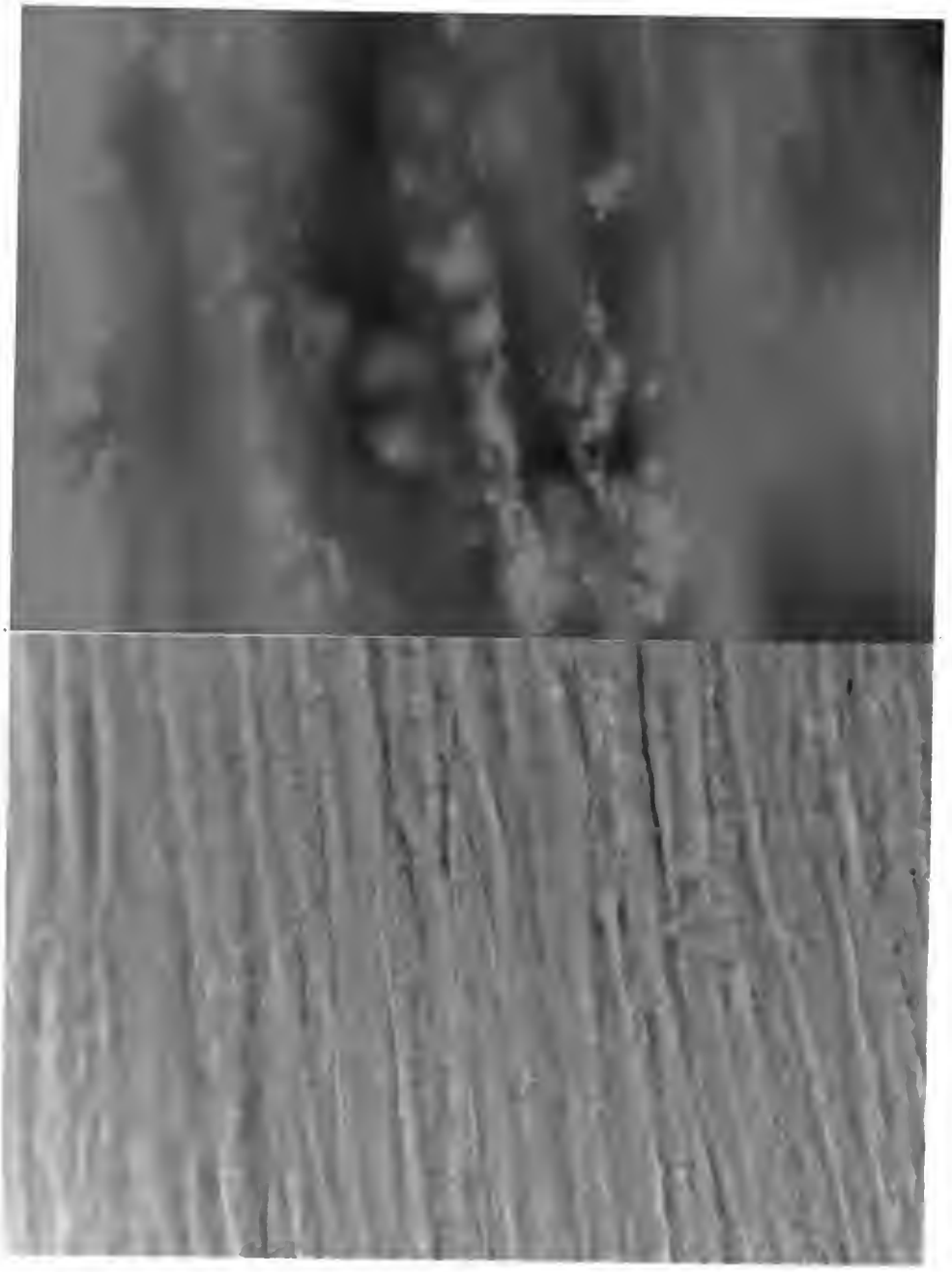

Fig. 3-10. Localization of P1 in epidermal tissue from stems of watermelon infected with ZYMV-FL/AT. Antiserum to the P1 of ZYMV-FL/AT (rabbit no. 1181, collection date 3-17-92)was used as the detecting antibody, and tissues were stained with Rhodamine-protein A and photographed with epifluorescence optics. Note the specific fluorescence of granular aggregates. Magnification =1,714 X. A, tissue photographed with epifluorescence optics; B, same field of view photographed with visible light.

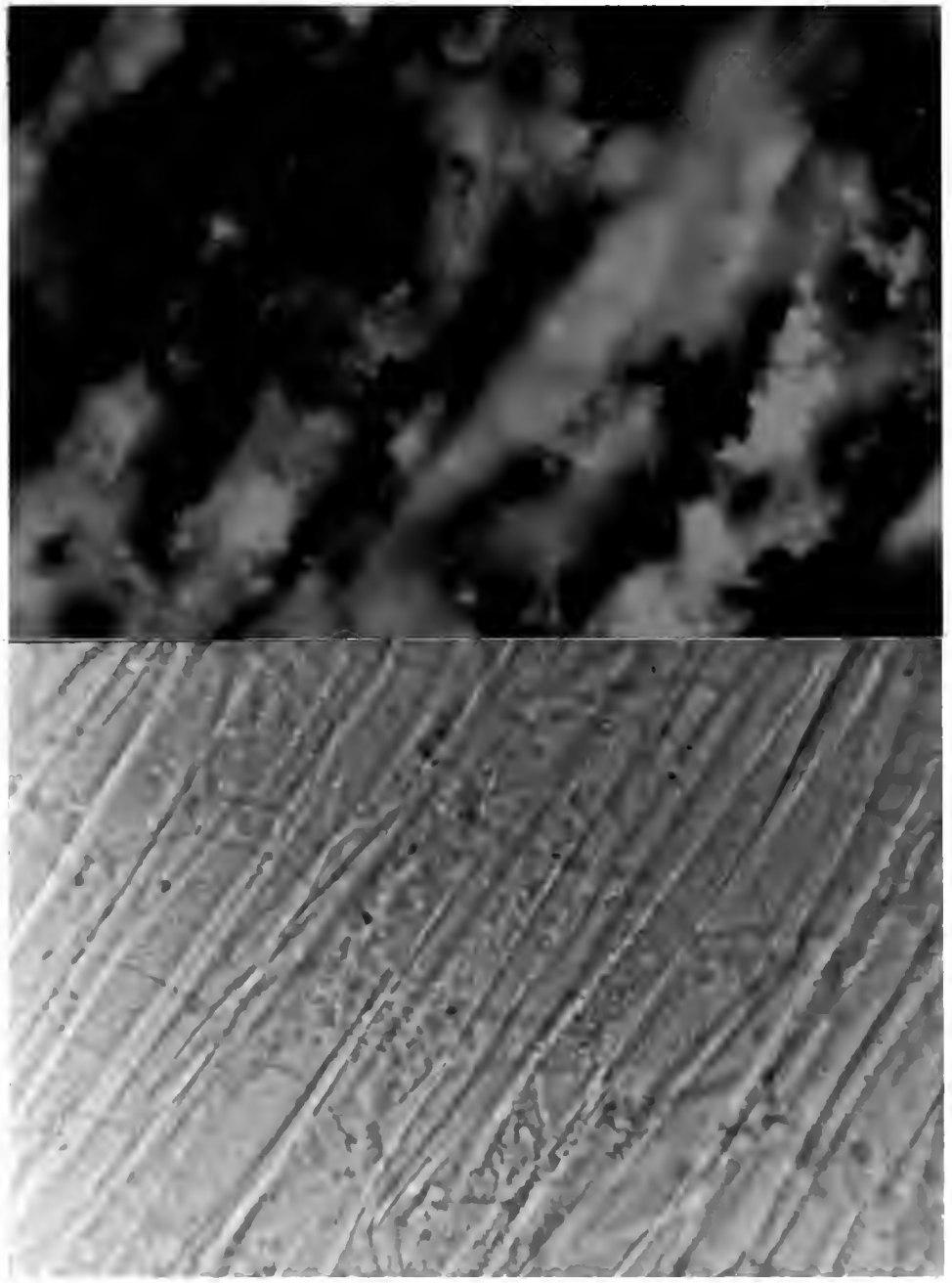

Fig. 3-11. Epidermal strips of watermelon tissue infected with ZYMV-FL/AT treated with preimmune serum, stained with Rhodamine protein A. Magnification =1714 X. A, tissue photographed with epifluorescence optics; B, same field of view photographed with visible light.

A

B

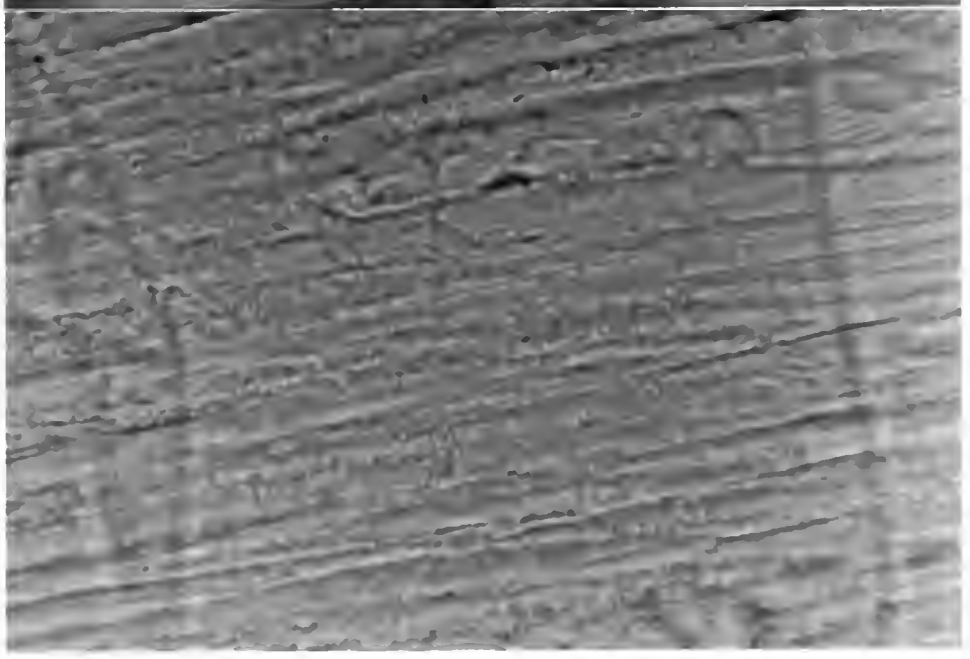

Fig. 3-12. Aggregates of P1 protein in watermelon stem epidermal tissue infected with ZYMV-RU treated with antiserum to the P1 of ZYMV-FL/AT (1181, collection date 3-17-92), stained with Rhodamine-protein A. Note granular aggregates are clearly visible, but do not fluoresce. Magnification =1714 X. A, tissue photographed with epifluorescence optics; B, same field of view photographed with visible light.

Fig. 3-13. Epidermal strips from mock-inoculated watermelon tissue treated with antiserum to the P1 of ZYMV-FL/AT (1181) and stained with Rhodamine-protein A. Note absence of fluorescent granular bodies. Magnification =1714 X.

Table 3-3. Summary of reactions of antisera to the P1 of ZYMV-FL/AT and ZYMV-RU in immunofluorescence tests.

	Reactivity[a]		
Isolate[b]	Antiserum to P1 of ZYMV-FL/AT	Antiserum to P1 of ZYMV-RU	Preimmune serum
ZYMV-FL/AT	+	-	-
ZYMV-SV	+	-	-
ZYMV-RU	-	+	-
FC-3182	+	+	-
Mock	-	-	-

[a] Reactivity determined as positive (+) by the presence of yellow, fluorescent aggregates in plant cells. Lack of reactivity (-) determined by absence of fluorescent aggregates.

[b] The isolates indicated were inoculated to watermelon plants.

Mock=mock-inoculated control plants.

Discussion

As was demonstrated in Chapter 2 for five ZYMV isolates, variability exists in the P1 nt and deduced amino acid sequences. Results from serological assays in immunoblots are in agreement with the sequence data. For example, the larger size seen in the nt and amino acid sequence of ZYMV-MD is reflected in the larger size of the P1 protein observed in western blots. The slightly smaller size of the P1 from ZYMV-SV in western blots was also reflected in a slightly smaller size of its respective gene in PCR analysis. The amino acids differences, reflecting different charges and polarities, may also influence the migration of P1 in SDS-PAGE.

Among the ZYMV isolates in this study, heterogeneity in the size of P1 was seen, as well as the presence of degradation products and differences in the capacity to fully process the cleavage site between P1 and HC/Pro. The size differences seen between ZYMV-FL/AT, ZYMV-SV, and ZYMV-MD P1 proteins were consistent regardless of the percent acrylamide, or of the plant host infected. The low nt (60%) and amino acid (70%) sequence homology between P1 of ZYMV-RU and ZYMV-FL/AT is reflected in the weak reactivity of the ZYMV-RU isolate in western blots when tested against antiserum to the P1 of ZYMV-FL/AT.

The P1 antiserum produced to ZYMV-FL/AT was specific for ZYMV. It neither reacted with extracts from plants infected singly with any of several other viruses that infect cucurbits, nor with extracts from noninfected cucurbit tissues. The ZYMV-FL/AT P1 antiserum precipitated a product of the predicted mw (ca. 34kDa) for P1 from WG translation products, with a smaller product of ca. 25-kDa. The small product may correspond to a similar sized product from infected tissue extracts seen in western blots.

Antiserum to P3 of ZYMV-FL/AT was not produced due to the toxicity of P3 to E. coli which precluded its production in vitro. Toxicity of P3 in E. coli has been reported with two other potyviruses (V. Doljas, E. Rodriguez-Cerezo, personal comm.). It was indicated by Rodriguez-Cerezo and Shaw (1991) that two regions on the P3 protein of TVMV were possible membrane spanning regions. These regions may somehow be involved in the toxicity of P3 to E. coli.

Antiserum to the P1 of ZYMV-FL/AT distinguished differences among ZYMV isolates. These differences were reflected in size variation in the P1 protein, possible breakdown products for some isolates, and incompletely processed polyprotein. Antiserum to the P1 of ZYMV-RU also showed differences in the size variation of the P1 protein like that seen with antiserum to the P1 of ZYMV-FL/AT. Antiserum to the P1 of ZYMV-RU also detected antigenic differences in western blots by lack of reactivity with some

ZYMV isolates. The ZYMV-RU P1 antiserum (rabbit no. 1186, collection date 6-10-92) reacted with 16 of 22 ZYMV isolates tested. This lack of reactivity seen may be due to titer differences between isolates, to limited sequence homologies, or differences in extractability of the P1 protein among isolates.

Immunofluorescence tests indicate that aggregation occurs with the P1 protein in infected plant tissues. This is shown by fluorescing aggregates in epidermal tissues from infected watermelon when treated with homologous P1 antisera. Pumpkin tissues were used in preliminary tests, but watermelon was a better host for sampling of epidermal strips. The antisera to P1 of ZYMV-FL/AT reacted with its respective isolate and with ZYMV-SV, but not with ZYMV-RU. Antiserum to P1 of ZYMV-RU reacted with its homologous antigen but not with ZYMV-FL/AT or with ZYMV-SV, in agreement with western blot analyses. Both the ZYMV-FL/AT P1 and ZYMV-RU P1 antisera reacted with FC-3182. This isolate also reacted strongly to both P1 antisera in western blots. Thus, isolate FC-3182 must have epitopes in common with both P1 from ZYMV-FL/AT and ZYMV-RU.

CHAPTER 4
SUMMARY AND CONCLUSIONS

Clones representing all portions of the ZYMV genome were obtained by the combined use of oligo dT primers, random primers, and a primer corresponding to the CI region of the genome. The nt sequences for the P1 of five ZYMV isolates were compared, three of which were sequenced in this study. A high nt and deduced amino acid homology was seen between four ZYMV isolates, including three from Florida and one from California. However, an isolate from Reunion Island had a low (60%) nt homology compared to the other ZYMV isolates from Florida and California. In contrast, the AI and P3 of ZYMV-RU had 84-88% nt sequence homologies compared to ZYMV-FL and ZYMV-CA. In addition, the sequence of the CP of ZYMV-RU is 88% similar to that of ZYMV-CA (Baker et al., 1991b). According to the criteria set forth by Shukla et al. (1991) the nt sequence homology of P1 from ZYMV-RU compared to the P1 of other ZYMV isolates (60%) classify it as a distinct potyvirus, while the sequence homologies of other regions of the ZYMV-RU genome (<90%) are not quite as high as that expected to be considered an isolate of the same virus.

The additional 18 nts in the 5'-terminus of the P1 gene of ZYMV-MD, coding for six additional amino acids, would account for the slightly larger size of the P1 PCR product seen for the ZYMV-MD isolate in agarose gels. This also accounts in part for the larger size of ZYMV-MD seen in western blots using antiserum to P1 of ZYMV-FL/AT.

Nucleotide sequence homologies between the P1 of ZYMV-FL/AT and other potyviruses including TEV, PVY^N, TVMV, PPV, and PSbMV ranged from 37% to 42%. These homologies also were lower than those for the AI (50-52%) and P3 (43-45%) compared to ZYMV-FL/AT. These data are in agreement with Shukla et al. (1991) in that the percent homology between distinct potyviruses is low, and that P1 is less conserved than the AI or P3 encoding region.

The N-termini of P1 proteins of the five ZYMV isolates in this study were less conserved than their C-termini. The additional six amino acids of ZYMV-MD were inserted directly following the methionine. The histidine missing in the ZYMV-SV is also in the N-terminus. Most of the changes in amino acids in both ZYMV-MD and ZYMV-SV are also in the N-termini.

The two amino acid residues in the C-terminus of P1 determined by Verchot et al. (1991) to be essential for protease activity are conserved in all ZYMV isolates in this study. The Tyr/Ser cleavage sites between P1 and HC/Pro are also conserved among the ZYMV isolates. Although the

consensus sequence, Leu-Val-Ile-Arg-Gly, was the same for all ZYMV isolates, it was slightly different from that published for 5 distinct potyviruses (Verchot et al., 1991).

The P1 proteins for both ZYMV-FL/AT and ZYMV-RU expressed from E. coli were insoluble, and thus were easily purified for antibody production. The P3 protein of ZYMV-FL/AT, however, was apparently toxic to E. coli in two different expression systems and thus was not produced in this study.

Antiserum specific to P1 of ZYMV-FL/AT reacted in western blots with all ZYMV isolates examined in this study, although the ZYMV-RU isolate either reacted weakly or gave no detectable reaction. According to the nt sequence, the P1 region of the ZYMV-RU genome is only 60% similar to that of ZYMV-FL/AT, with a 70% amino acid similarity. This may account for the low level of reactivity in western blots and lack of reactivity in immunofluorescence tests. Nucleotide and amino acid heterogeneity was seen among the P1 of five ZYMV isolates. Heterogeneity was also observed in western blots among the P1 proteins of 22 ZYMV isolates tested against ZYMV-FL/AT P1 antiserum. There were differences in the size of the P1 protein produced, in the presence of breakdown products, and in the incomplete processing of the P1 and HC/Pro polyprotein.

The antiserum to the P1 of ZYMV-FL/AT did not react with extracts from plants infected individually with three

other potyviruses, one cucumovirus, or one potexvirus. Antiserum to the P1 of ZYMV-RU did not react in western blots with the three potyviruses, or with the potexvirus, but was not tested against CMV.

The specificity seen for the ZYMV-FL/AT P1 antiserum in western blots was also seen in indirect immunofluoresence tests in epidermal strips from watermelon. The P1 antiserum reacted with ZYMV-FL/AT, and ZYMV-SV, but not with ZYMV-RU or with healthy (mock inoculated) watermelon tissues. The lack of reactivity of antiserum to ZYMV-FL/AT P1 protein with ZYMV-RU infected tissues in immunofluorescence is not surprising due to the weak reactivity seen in western blots and to the limited sequence homology between these two isolates. Immunofluorescence tests using antiserum to the P1 of ZYMV-RU showed reactivity with ZYMV-RU but not with ZYMV-FL/AT or ZYMV-SV. These results are reflected in the lack of reactivity seen with these isolates in western blots using antiserum to ZYMV-RU P1 as a probe. The FC-3182 isolate, which reacted with the antisera to both ZYMV-FL/AT and ZYMV-RU P1 in western blots, likewise reacted with both antisera in immunofluorescence tests, indicating the presence of shared epitopes among the P1 of ZYMV-FL/AT, ZYMV-RU, and FC-3182 isolates. The P1 proteins of ZYMV-FL/AT, ZYMV-SV, ZYMV-RU, and FC-3182 were detected in infected tissue with the immune serum as amorphous, particulate aggregates in the cytoplasm. Further analysis

by electron microscopy is needed to determine the precise morphology of the aggregates and their possible association with other viral proteins or with host components.

The antisera developed in this study have been useful for detecting variability of ZYMV isolates by immunoblotting. Antiserum to the P1 of ZYMV-FL/AT was specific to ZYMV isolates, and showed that there are differences in the size of P1 proteins and occurrence of related products among isolates. The antiserum to P1 of ZYMV-RU showed differential reactivity to certain isolates of ZYMV, indicating possible antigenic differences among ZYMV isolates, differences in titer among isolates, or differences in extractability among ZYMV isolates.

The P1 amino acid sequence showed heterogeneity among ZYMV isolates in this study, with the N-terminus being less conserved than the C-terminus. These traits are noted for other potyviruses (Verchot et al., 1991), supporting the speculation that P1 may be associated with virus-host interactions. This may be true in particular for the N-terminus of P1 which is more variable than the C-terminus. Further studies to elucidate this possible involvement of P1 in host response might involve the use of full length infectious transcripts. With these transcripts the P1 from different isolates could be interchanged to determine the possible effects on the host response. Alternatively, mutants in the P1 region could be used for this purpose.

Because there is considerable biological variation available in naturally occurring ZYMV isolates, the former study may be more meaningful.

Further studies on P3 should include cloning portions of the protein separately to avoid the possibility of cloning toxic regions which may disrupt the bacterial cell processes. The P3 is also an interesting protein for study, since little is known about its function.

APPENDIX
MONOCLONAL ANTIBODIES TO THE CAPSID PROTEIN OF ZUCCHINI YELLOW MOSAIC VIRUS

Introduction

Researchers at the University of Florida, the Volcani Center in Israel, and at the USDA in Orlando, Florida, have been coordinating efforts on potyviruses which infect the Cucurbitaceae. These studies involve comparison and distinction of isolates using differential plant hosts, development of polyclonal antisera used in immunodiffusion and ELISA tests, in vitro translational studies, aphid transmission, characterization of nonstructural proteins, and development of monoclonal antibodies (MAbs). As an early part of the research for the present study, several MAbs to the capsid protein (CP) of ZYMV were evaluated for their ability to distinguish and differentiate isolates of ZYMV. One MAb to watermelon mosaic virus-2 (WMV-2) was also evaluated for its diagnostic potential for WMV-2 isolates. Two MAbs to papaya ringspot virus-type W (PRSV-W) obtained in another study (Baker et al., 1991a) were combined and evaluated for their diagnostic potential along with the MAbs to ZYMV and WMV-2. The following materials and methods

detail procedures to develop and evaluate MAbs to ZYMV and WMV-2.

Materials and Methods

Immunization Protocol

In all cases, four Balb/c mice were immunized on day one with a subcutaneous (SC) injection of 50 μg purified virus in 0.5 ml distilled water, emulsified in an equal volume of Freund's complete adjuvant (FCA). The subsequent injections, except for the final one, also 50 μg, were administered intraperitoneally (IP), emulsified in an equal volume of Freund's incomplete adjuvant (FIA). One mouse was selected for a fusion based on results from a test bleeding. It was immunized with 25 μg in 0.5 ml water without adjuvant directly into the tail vein (intravenous, IV). If the entire volume could not be injected into the tail vein the remainder was injected IP. Three to five days after the IV injection the mouse was sacrificed for a fusion.

Two additional immunization protocols were used in this study. One will be referred to as *in vivo* immunization and involved the protocol described above. An additional *in vitro* fusion protocol was used as outlined by Boss (1986), Weigers et al., (1986), and S. Zam (personal comm.). Two mice were either previously immunized as described above or not previously immunized. In either case the final

immunization was administered to the two combined excised spleens in culture on the day the mice were sacrificed. This in vitro boost consisted of 25 μg antigen and 20 μg/ml of the adjuvant N-acetyl muramyl L-alanyl-D-isoglutamine (Sigma Chemical Co., St. Louis, MO). The fusion was performed three to five days later.

The immunization schedule for a poorly aphid transmissible (PAT) isolate of ZYMV involved injections on day one, 21, and 28, followed by a fusion on day 31. For an aphid-transmissible isolate of ZYMV from Florida (ZYMV-FL/AT), mice were immunized individually with both whole virus or capsid protein (CP). The immunization schedules for the ZYMV-FL/AT isolate preparations were: day one, day 30, day 37, (IV, IP, or an immunization in vitro), with the fusion on day 40. The immunization schedule which was effective in production of MAbs for WMV-2 (isolate FC-1656) was: day one, day 21, day 83, day 147, day 167 (IV and IP), with the fusion on day 170.

Fusion Protocol

The fusion procedures followed are similar to those outlined by Galfre and Milstein (1981). The mouse was sacrificed by placing it in a closed container with a small piece of dry ice for approximately 30 sec, and the spleen was excised and gently mashed through an 80-mesh sieve, and placed in 5 ml of serum-free Dulbecco's modified eagle medium (DMEM, Gibco Laboratories, Grand Island, NY). The

SP2/O myeloma cell line, obtained from the University of Florida Hybridoma Laboratory and used as the fusion partner, was maintained in a logarithmic growth phase in DMEM-F, supplemented with 10% fetal calf serum, 1 mM L-glutamine, 1% penicillin/streptomycin, and 0.001 mg/ml amphotericin B (fungizone, Sigma Chemical Co., St. Louis, MO). Both the spleen cells and the SP2/O cells were washed twice by centrifugation at 2,000 X *g* for 7 min in serum free DMEM, and were combined at a ratio of one part SP2/O cells to two parts spleen cells, respectively. They were centrifuged together a final time and were resuspended in 50% PEG [mw 1500, in 75 mM HEPES (pH 8.0)] over a period of 15 sec. The cell suspension was swirled in a 37 C water bath for 30 sec, and incubated for 90 sec at 37 C. One ml of serum-free DMEM was then added over 30 sec, swirled 30 sec, followed by the addition of 10 ml DMEM over 30 sec and incubation for 5 min in a 37 C water bath. The solution was then centrifuged and the pellet resuspended in HAT selective media containing 20% fetal calf serum to give a final concentration of 5 X 10^8 cells/ml. The HAT medium contained DMEM-F with 20% fetal calf serum and 1 ml HAT/50 ml of media (5 mM hypoxanthine, 0.02 mM aminopterin, 0.8 mM thymidine, Sigma Chemical Co., St. Louis, MO). These samples were plated out at 100 μl/well in 96-well plates, and placed in a 37 C incubator with an atmosphere of 6% CO_2 and 90% humidity. Cultures were fed with 50 μl/well of HT medium (DMEM-F with

hypoxanthine and thymidine, but without aminopterin) 7 days after fusion and then screened serologically for antibody production as colonies developed.

Screening Procedures

All hybridoma cultures were screened against their homologous virus in both an antibody-trapped and plate-trapped indirect enzyme linked immunosorbent assay (ELISA) (Halk, 1986), using purified virus and extracts from both healthy and infected Cucurbita pepo L. 'Small Sugar' pumpkin tissue. The antibody-trapped indirect ELISA consisted of the following steps: (1) rabbit antiserum to ZYMV at 1/1000 dilution in coating buffer, pH 9.6, was placed in Immulon II microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) at 75 or 100 μl per well. Plates were incubated at 4 C overnight or at 37 C for 1 hr. Each step was followed by three 5 min washes in 0.1 M phosphate buffered saline (PBS), pH 7.4, with 0.5% Tween-20 (PBST); (2) antigen was added at 50 or 75 μl/well, diluted in PBST at 1/1000 dilution (pure virus) or 1/10 (one part plant tissue triturated in nine parts PBS triturated in a mortar and pestle, and expressed through cheesecloth). Incubation was at 37 C for 1 hr; (3) MAb tissue culture supernate or ascites was diluted appropriately and added at 50 μl/well, and incubated at 37 C for 1 hr; (4) the enzyme conjugate, goat anti-mouse IgG and IgM alkaline phosphatase was added at 1/1000 dilution each in conjugate buffer (PBST with 2% PVP-40 and 0.2% ovalbumin)

at 50 μg/well, at 37 C, for 1 hr; (5) The substrate, p-nitrophenylphosphate (PNPP, Sigma Chemical Co., St. Louis, MO) was added at 1 mg/ml using 50 μl/well. Plates were read with a Biotek automated microplate reader EL309 (Bio-Tek Instr., Winooski, VT) every 15 min as reactions developed. The plate-trapped indirect ELISA consisted of the same steps as above, omitting the first step and with the antigen dilutions made in coating buffer. Ascites were usually diluted 1/1000 to 1/10,000 in PBS, and tissue culture supernates were diluted at 1/100 to 1/1000 depending on the reactivity of the clone.

Two MAbs to PRSV-W, which in combination reacted with 15 PRSV-W isolates, were kindly provided by C.A. Baker.

Cloning and Ascites Production

The most promising cell lines were selected for cloning, increased in large volumes, and used for ascites production. Each culture was cloned two to three times by limiting dilution. Isotyping was done by using a Zymed Monoclonal antibody ID kit (Zymed Lab Inc., San Francisco, CA). Ascites were produced by injecting at least 1 X 10^6 cells into Balb/c mice which had been primed 10-14 days earlier with pristane (0.5 ml, IP). Ascites were collected from five days to three weeks later using an 18-gauge needle, and clarified by centrifugation.

Results

ZYMV-specific MAbs to the CP and whole virus were obtained from the PAT and AT isolates. One MAb of WMV-2 was selected for serological analysis of WMV-2 isolates. Reactions in ELISA were considered positive if the absorbance at 405 nm was three times the healthy reading and greater than 0.1.

One MAb to ZYMV, MAb-Z1, reacted with extracts from plants infected with all 15 ZYMV isolates tested (10 from Florida, three from France, one from Italy, and one from Reunion Island) (Fig. 1). Reactions to the Reunion Island isolate were usually low in absorbance value (A_{405} less than 0.5). MAb-Z1 did not react with extracts from noninoculated pumpkin, or with those from PRSV-W, WMV-2, a distinct cucurbit potyvirus (2932), or cucumber mosaic virus (CMV) (Fig. 1). The MAb-Z1 (clonal designation BB2-A2-D4) was derived from the PAT isolate of ZYMV and only reacted in antibody-trapped indirect ELISA tests, but not in plate-trapped ELISA tests.

MAb-Z2 (clonal designation AG7-D6-C6) was derived from the AT isolate of ZYMV and reacted in both antibody-trapped and plate-trapped ELISA (Fig. 2). This MAb reacted with neither extracts from noninoculated plants, nor with extracts from plants infected with PRSV-W, WMV-2, cucurbit potyvirus FC-2932, or CMV.

MAb-Z3 (clonal designation AD4-H11-D10), made to the CP of the AT isolate of ZYMV also reacted in both antibody and plate-trapped ELISA, and often very weakly to the Reunion Island isolate (Fig. 3). Weakly positive reactions to PRSV-W were seen. In plate-trapped ELISA this MAB reacted very weakly or not at all with the ZYMV-SV isolate from Florida.

MAb-Z4 (clonal designation DA3-B7-D4), derived from the CP of the AT isolate of ZYMV, reacted well in both antibody-trapped and plate-trapped ELISA (Fig. 4). This MAb also reacted to WMV-2 and CMV at low levels (0.167 and 0.303, respectively). These reactions are considered borderline between positive and negative, as often seen with some MAbs. Absorbance readings could be considered positive only if the reaction was allowed to proceed over approximately three hours.

MAb-Z5 (clonal designation BE7-F9-E10) made to the AT isolate of ZYMV was similar to MAb-Z4, in that both reacted in antibody- and plate-trapped ELISA, and both produced weakly positive absorbance readings to WMV-2 and CMV (Fig. 5).

MAB-Z6 (clonal designation FD5-B7-G6) was produced to the AT isolate of ZYMV and reacted in both types of ELISA tests (Fig. 6). Reactions to the Reunion Island isolate were usually weak, but this was also variable, and could sometimes be considered positive. This MAb also gave low, but slightly positive readings with WMV-2 and CMV.

MAb-W (clonal designation FD1-B5-B5) to WMV-2 reacted specifically in antibody-trapped ELISA to WMV-2 isolates including 14 from Florida, two from California, and one each from New York and New Zealand (Fig. 7). A distinction was made, however, between two sources of the ATCC isolate of WMV-2. Both sources, PV-27 and PV-27/V619 presumably came from the same original accession, except the latter was obtained several years earlier and was maintained in greenhouse culture in Florida in C. pepo. All WMV-2 isolates reacted with polyclonal anti-WMV-2 sera in indirect ELISA and immunodiffusion. MAB-W did not react with any ZYMV or PRSV-W isolate.

MAbs-1, -2, -3, and -4 were all of the IgM isotype. MAbs -5 and -6 were of the IgG1 isotype. MAb-W was the IgG1 isotype. All MAbs were of the kappa light chain subclass.

Polyclonal antisera to ZYMV reacted with all isolates in plate-trapped ELISA and also cross-reacted with PRSV-W (A_{405} of 0.871), WMV-2 (0.251), and cucurbit potyvirus FC-2932 (0.314) (Fig. 8). This antiserum did not cross-react with CMV or with noninfected tissue extracts. Conditioned medium and ascites from nonimmunized mice did not react with any virus isolate and reactions in both types of ELISA tests were less than 0.10.

Two MAbs to PRSV-W were used in combination to detect 17 PRSV isolates in both ELISA tests. These isolates included 15 from Florida, one each from New York,

California, Jordan, and Greece, the ATCC, one isolate of PRSV-T (Tigre), and one isolate of PRSV-P (papaya). These MAbs did not react with any WMV-2 or ZYMV isolate.

MAb-Z1, MAb-W, and the combined PRSV-W MAbs were evaluated together for their diagnostic potential. Three isolates of each virus were tested against a MAb to ZYMV, a MAb to WMV-2, and to the combination of two MAbs to PRSV-W (Fig. 9). There was no cross-reactivity between the three viruses and their respective antibodies, and no reactivity with CMV, zucchini yellow fleck virus (ZYFV), or with tissue extracts from noninoculated pumpkin.

Discussion

The MAb-Z1 used in antibody-trapped indirect ELISA detected all ZYMV isolates tested. A low absorbance reading was often seen with the Reunion Island isolate. This MAb may have a low affinity for that particular isolate, and this may be the explanation for some of the low readings consistently seen.

The MAb to WMV-2 also has value for diagnosis of WMV-2 isolates. It reacted with 19 of 20 WMV-2 isolates in antibody-trapped ELISA.

Although the two MAbs to PRSV-W individually were selective to particular PRSV-W isolates, in combination they reacted with all PRSV-W isolates tested.

Four MAbs, one to ZYMV, one to WMV-2, and two to PRSV-W in combination, were effective in detection of the three respective viruses of cucurbits. Together, these viruses represent major problems to the production of cucurbits wherever they are grown. These experiments show a diagnostic potential of MAbs after thorough screening in ELISA against a variety of virus isolates from several different areas of cucurbit production.

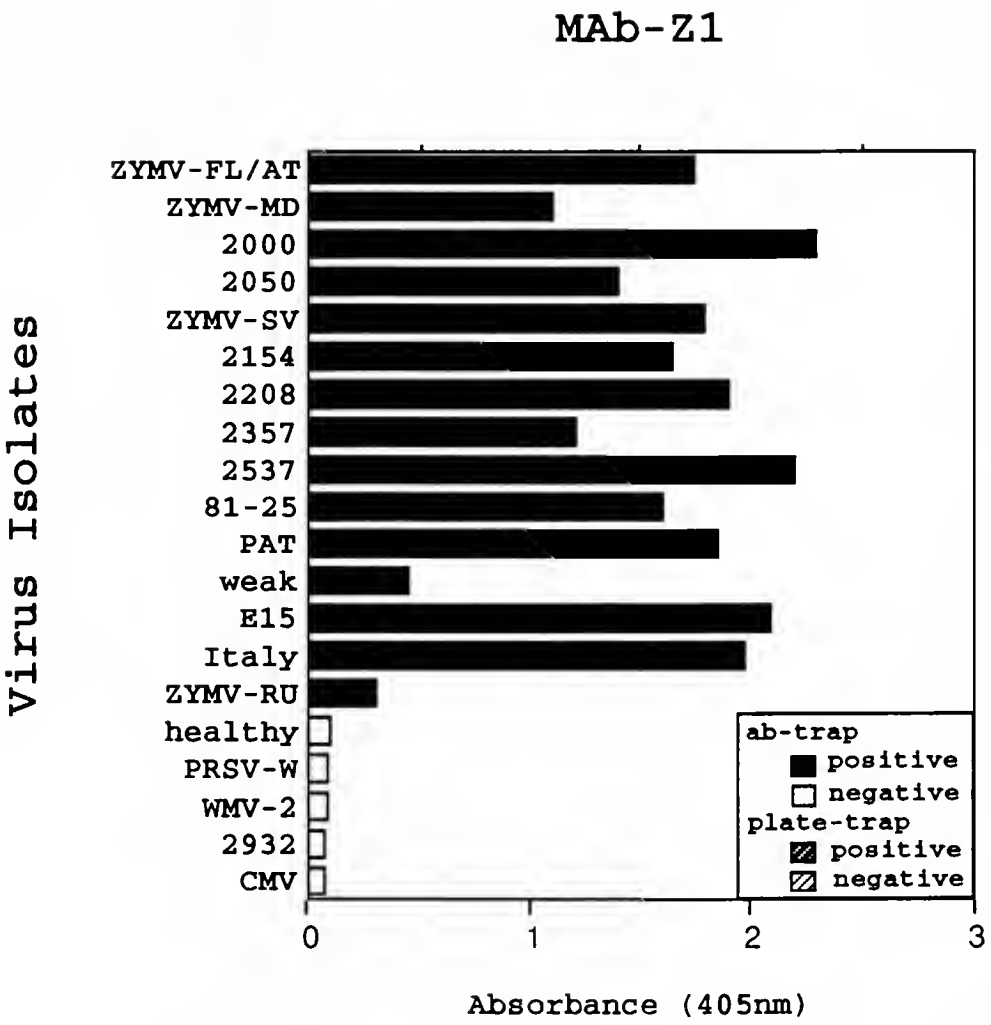

Fig.1. Reactivity of MAb-Z1 (BB2-A2-D4) to 15 ZYMV isolates and four cucurbit viruses in antibody-trapped indirect ELISA (ab-trap). Absorbance readings (405 nm) were taken three hrs after substrate addition and are an average of two wells. MAb-Z1 did not react in plate-trapped indirect ELISA.

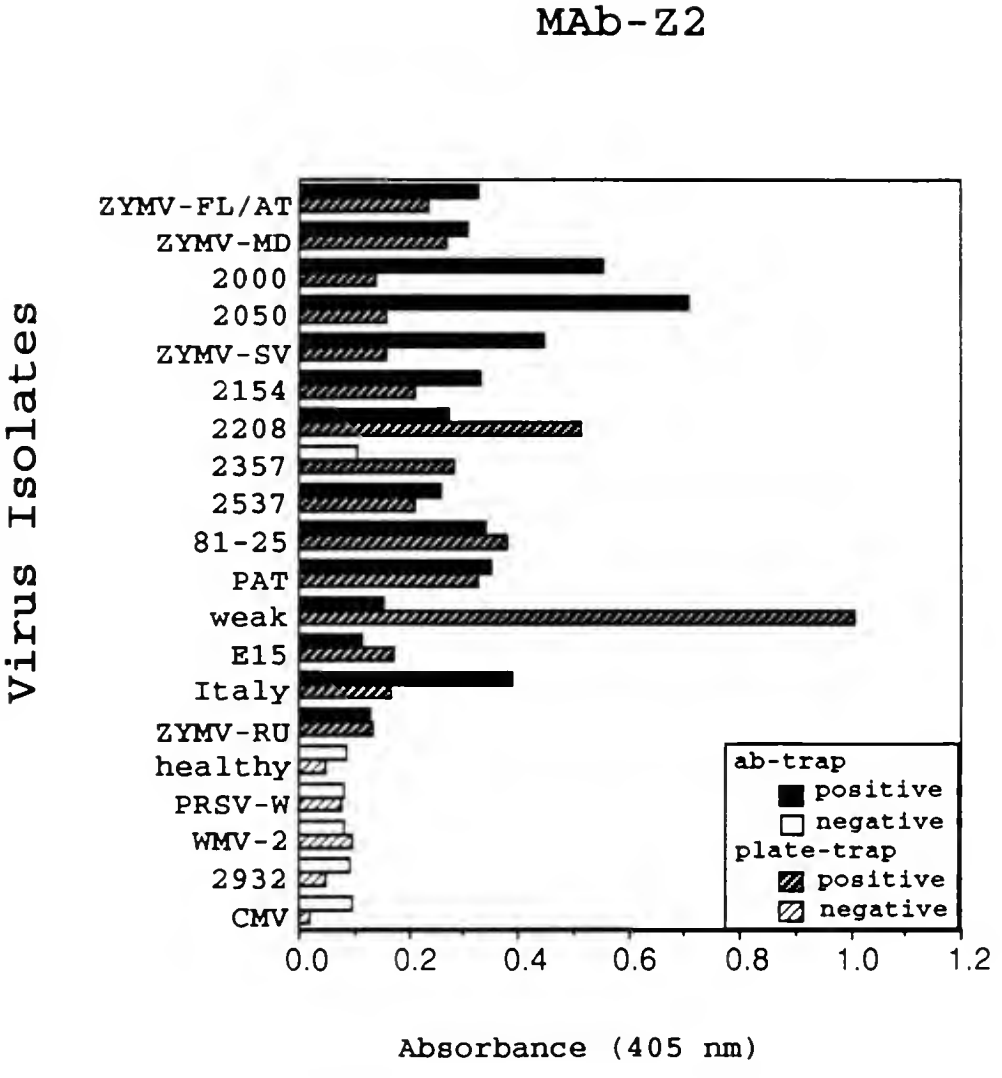

Fig.2. Reactivity of MAb-Z2 (AG7-D6-C6) to 15 ZYMV isolates and four cucurbit viruses in antibody-and plate-trapped ELISA. Absorbance readings (405 nm) were taken three hrs after substrate addition and are an average of two wells.

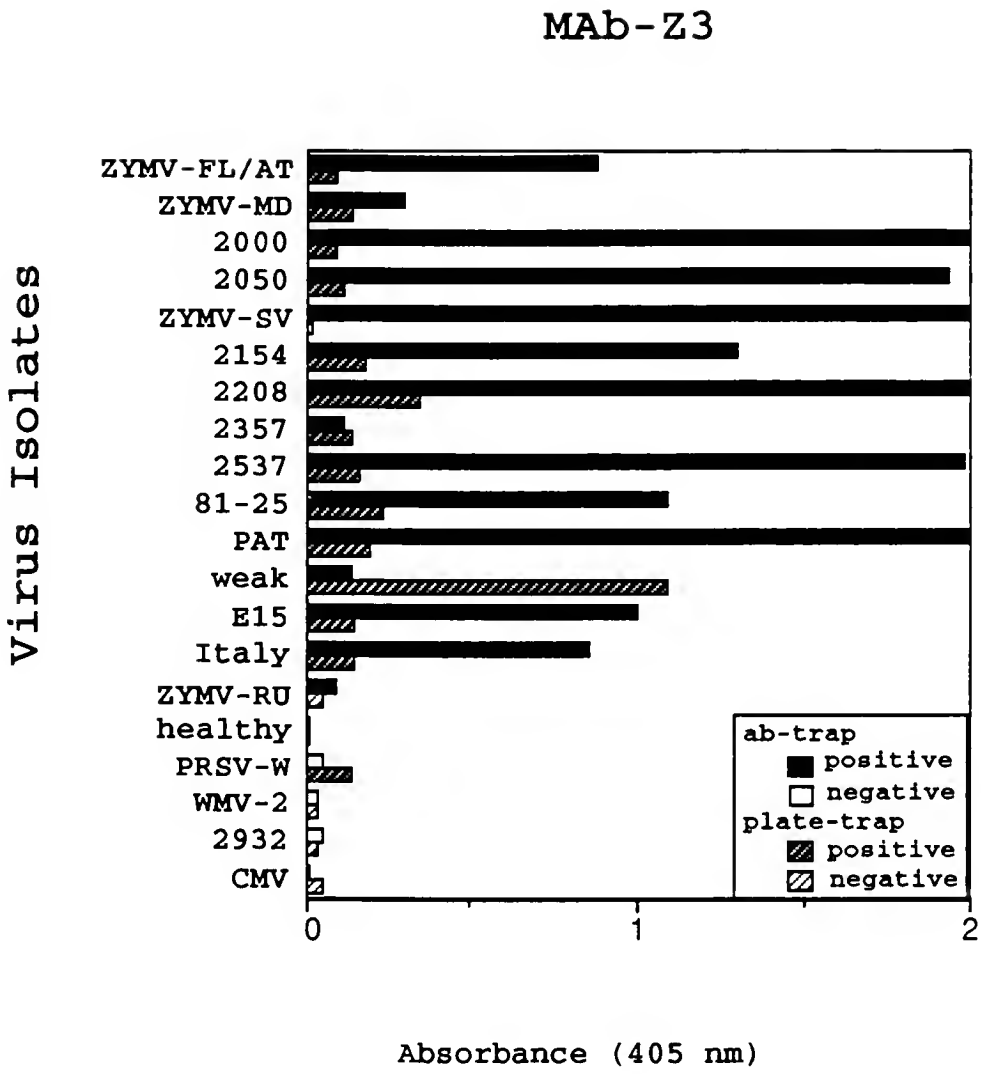

Fig. 3. Reactivity of monoclonal antibody MAb-Z3 (AD4-H11-D10) in antibody-trapped (ab-trap) and plate-trapped ELISA. Readings were taken three hrs after substrate was added and are an average of two wells.

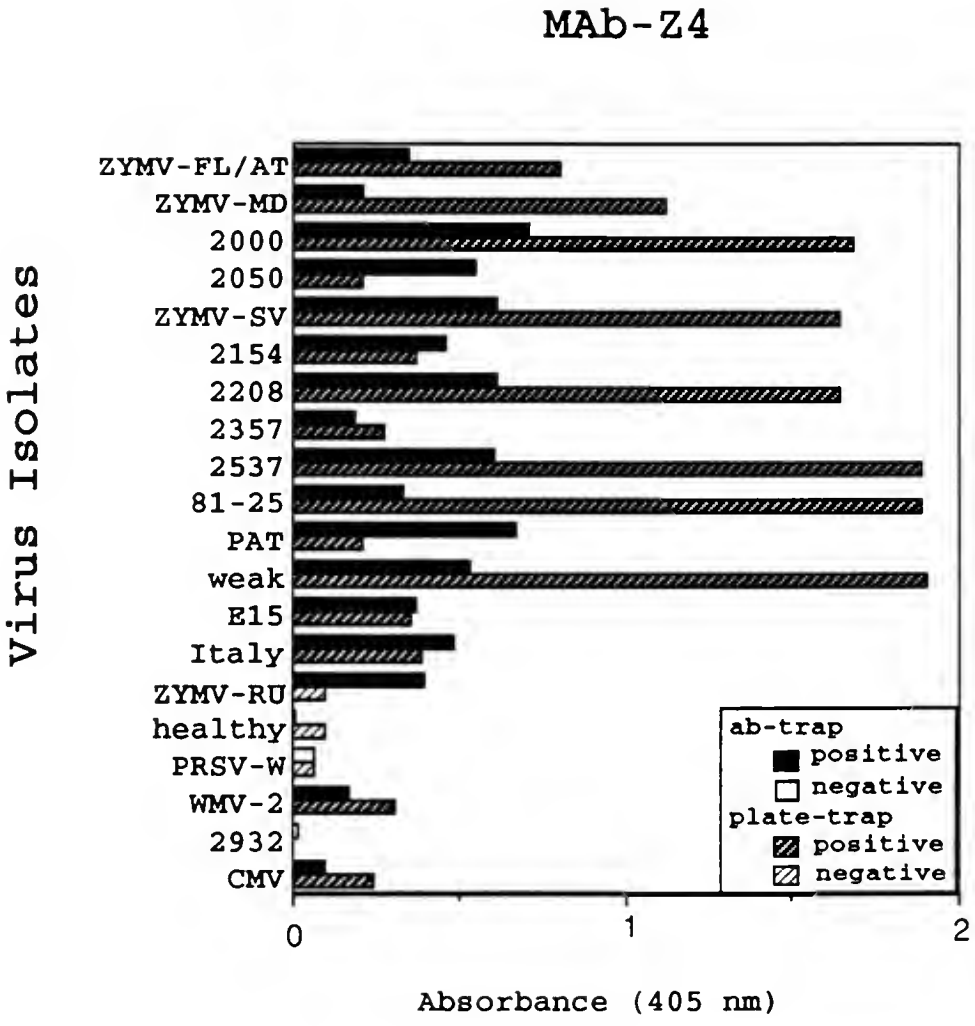

Fig. 4. Reactivity of MAb-Z4 (DA3-B7-D4) in antibody- and plate-trapped indirect ELISA to 15 ZYMV isolates. Absorbance readings (405 nm) were taken three hrs after substrate addition and are an average of two wells.

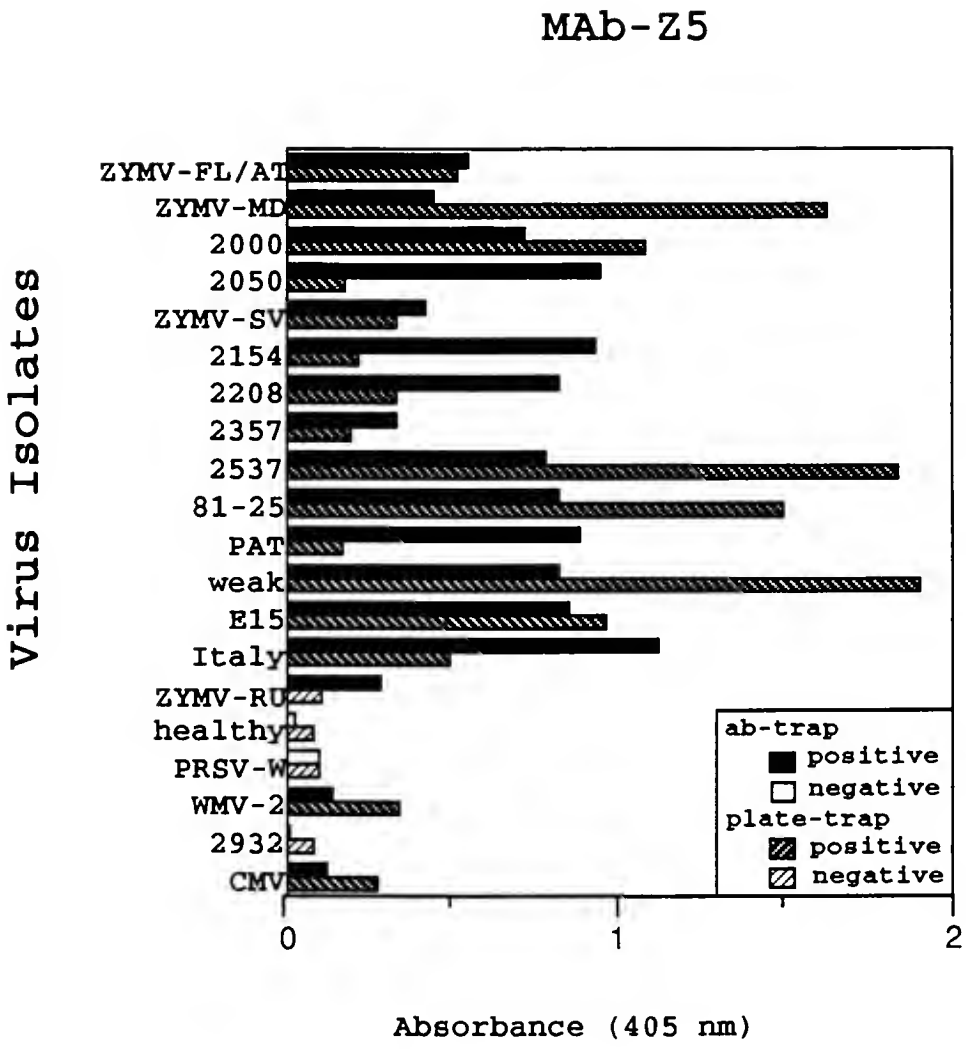

Fig. 5. Reactivity of MAb-Z5 (BE7-F9-E10) in antibody- and plate-trapped indirect ELISA to 15 ZYMV isolates. Absorbance readings (405 nm) were taken three hrs after substrate addition and are an average of two wells.

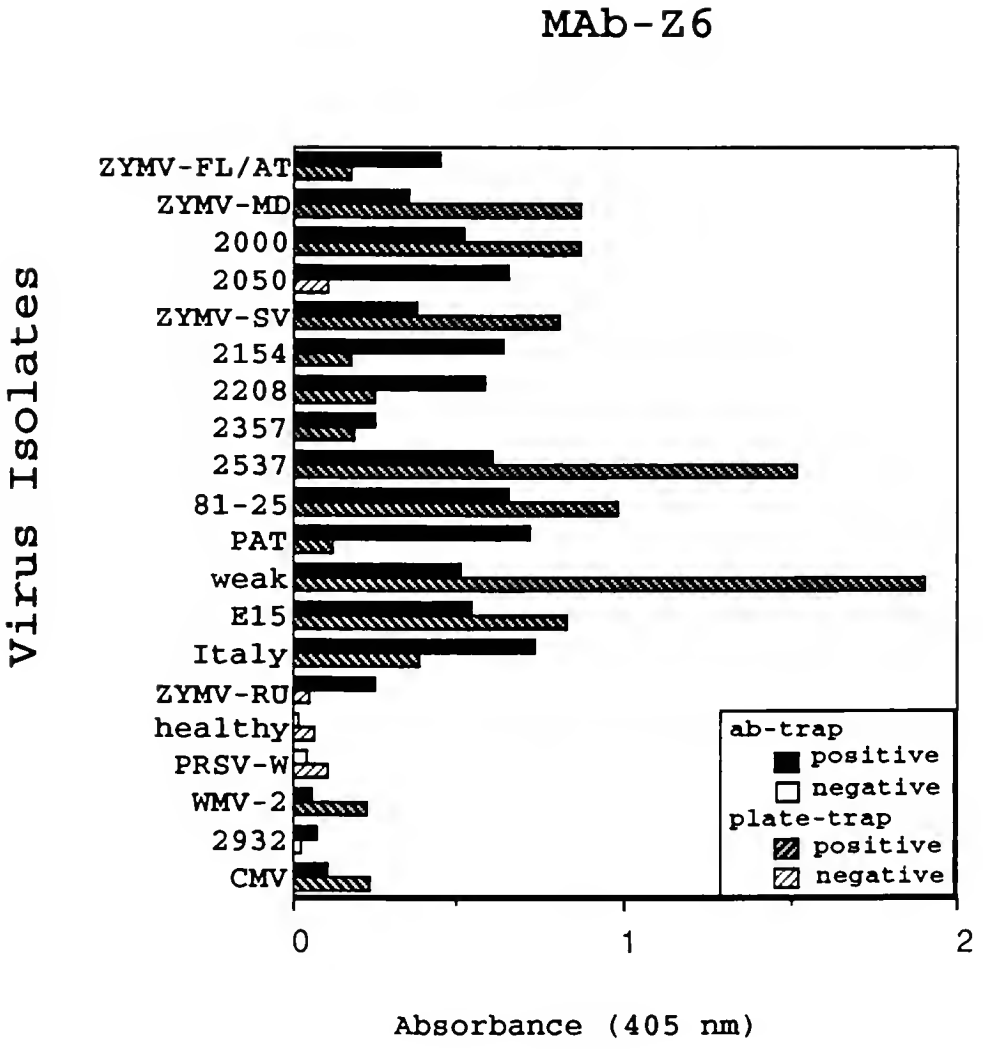

Fig. 6. Reactivity of MAb-Z5 (FD5-B7-G6) in antibody- and plate-trapped indirect ELISA to 15 ZYMV isolates. Absorbance readings (405 nm) were taken three hrs after substrate addition and are an average of two wells.

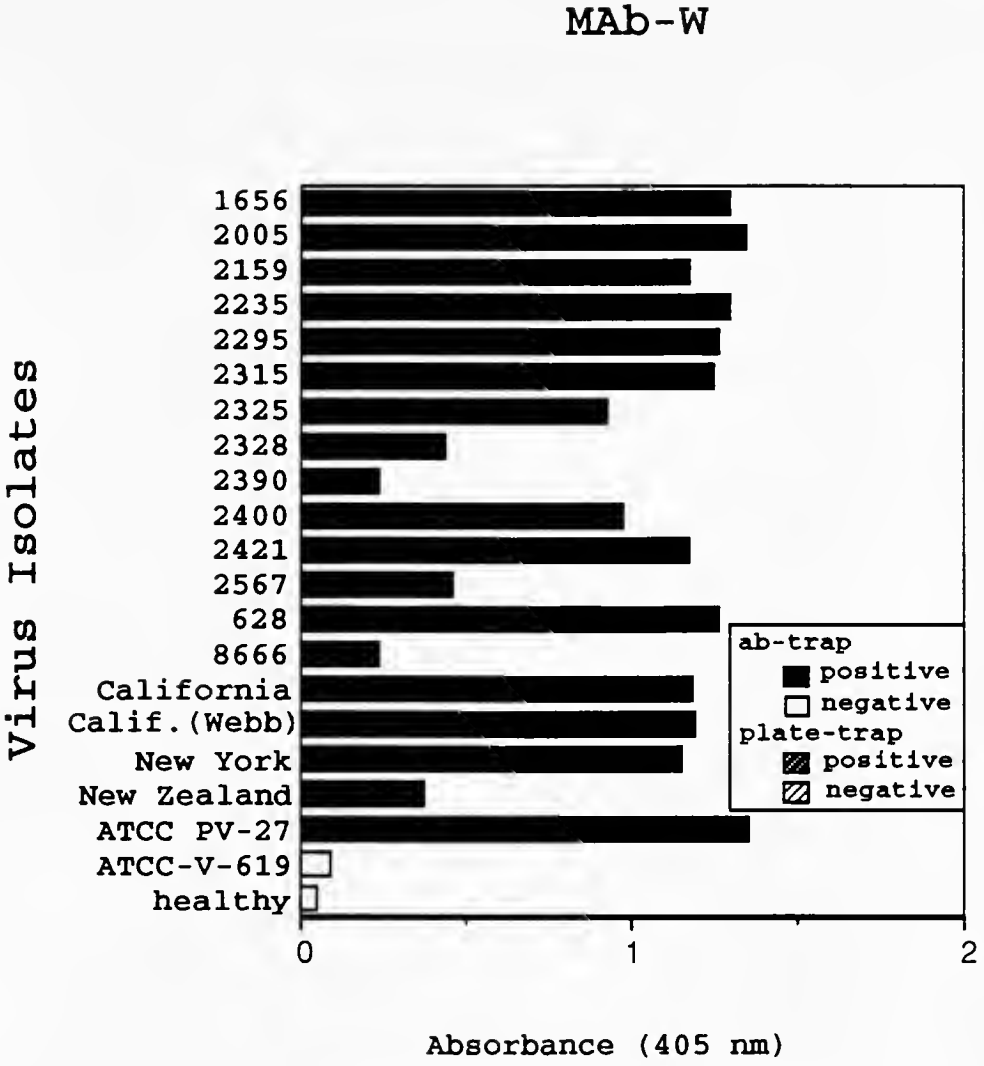

Fig. 7. Reactivity of monoclonal antibody MAb-W (FD1-B5-B5) to 20 isolates of WMV-2 in antibody-trapped indirect ELISA. Only one isolate of WMV-2, derived from an ATCC isolate, did not react with the antibody. Readings were taken three hrs after substrate addition and are an average of 2 wells. MAb-W did not react in plate-trapped indirect ELISA.

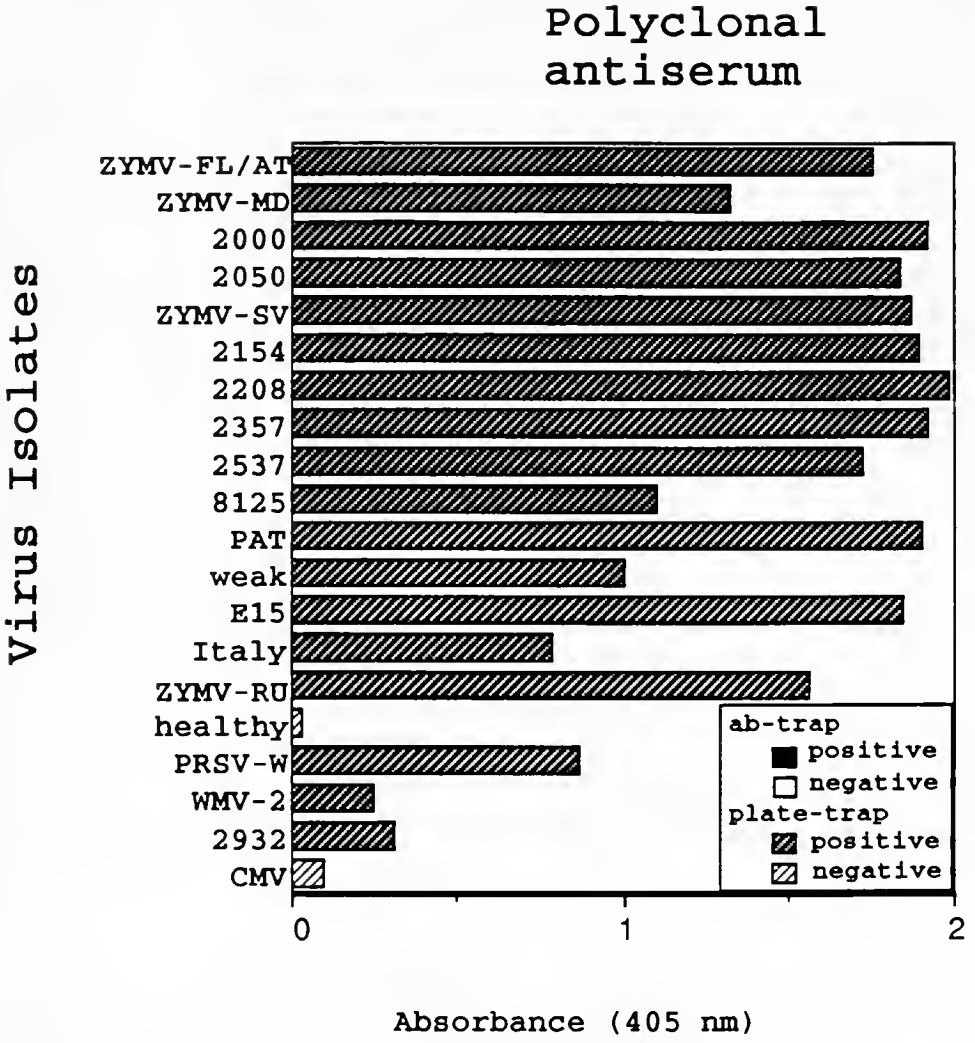

Fig. 8. Reactivity of polyclonal antiserum to the capsid protein of ZYMV (rabbit no. 1028) with ZYMV isolates and other cucurbit viruses in plate-trapped indirect ELISA. Note cross-reactivity with PRSV-W, WMV-2, and a distinct potyvirus, 2932. Conditioned medium and ascites from nonimmunized mice gave absorbance readings (A 405 nm) of less than 1.0 for all isolates in both antibody- and plate-trapped indirect ELISA (data not shown). This antiserum was not used in antibody-trapped indirect ELISA.

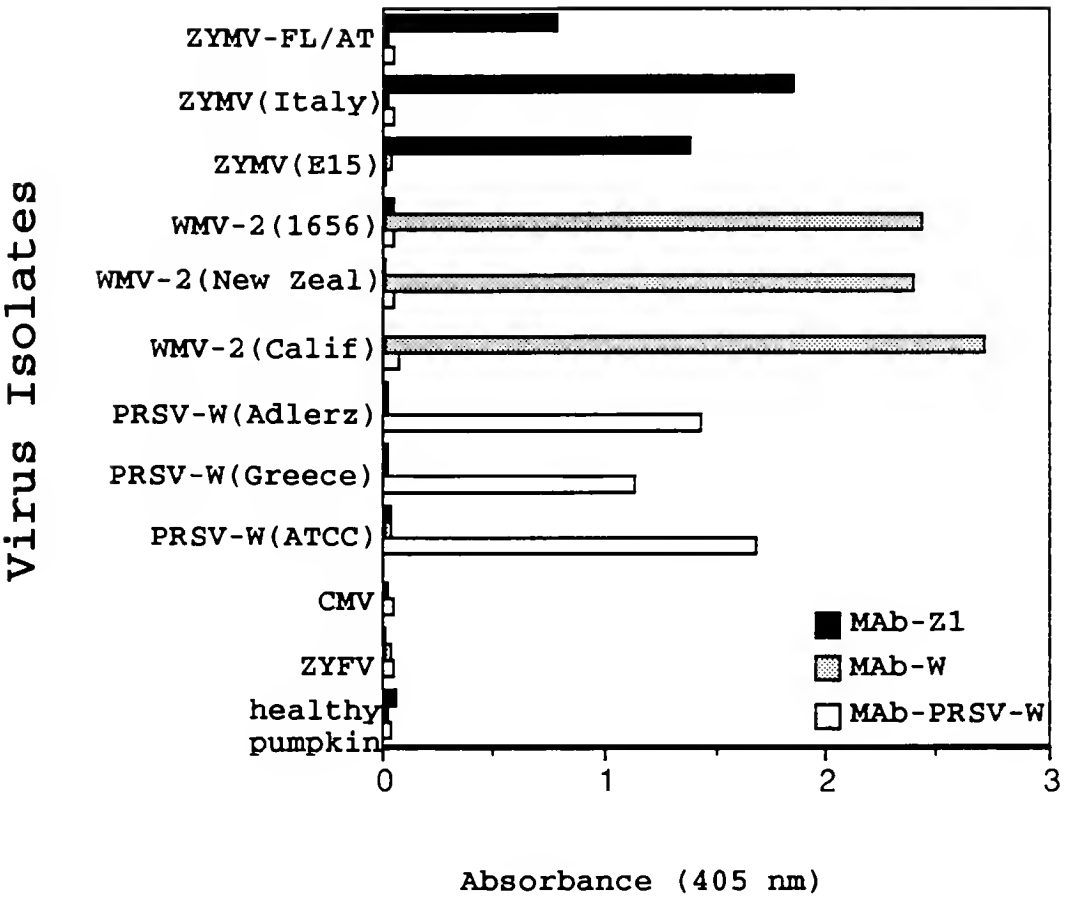

Fig. 9. Antibody-trapped indirect ELISA for diagnosis of ZYMV, WMV-2, and PRSV-W. Monoclonal antibodies used are MAB-Z1 to ZYMV (BB2-A2-D4), MAb-W to WMV-2 (FD1-B5-B5), and a combination of two monoclonals to PRSV-W (F3C-C10 and F21C-E4). Readings were taken after three hrs after substrate addition and are an average of 2 wells.

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BIOGRAPHICAL SKETCH

Gail C. Wisler was born in Indianapolis, Indiana, on Feb. 23, 1954. She graduated from the College of William and Mary in 1976 with a B.S. in biology. She started work at the Florida Department of Agriculture & Consumer Services, Division of Plant Industry (DPI) in the Bureau of Plant Pathology as a laboratory technician following graduation from William and Mary. In 1979 she began a M.S. program under the direction of F.W. Zettler in the Plant Pathology Department of the University of Florida, while continuing to work fulltime. She finished her M.S. in 1981 and continued to work at DPI, where she concentrated on plant viruses and related problems. She left DPI in 1986 to head the monoclonal antibody laboratory at the University of Florida Plant Pathology Department on a two year grant funded position for cucurbit potyviruses and geminiviruses. She started a Ph.D. program in September, 1988 under the direction of D.E. Purcifull, working on the molecular biology of zucchini yellow mosaic virus. She expects to receive her degree in August of 1992. Gail is a member of the American Phytopathological Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dan E. Purcifull, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ernest Hiebert
Professor of Plant Pathology

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F.W. Zettler
Professor of Plant Pathology

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Professor of Agronomy

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1992

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